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The basic neural circuitry for sexual behavior

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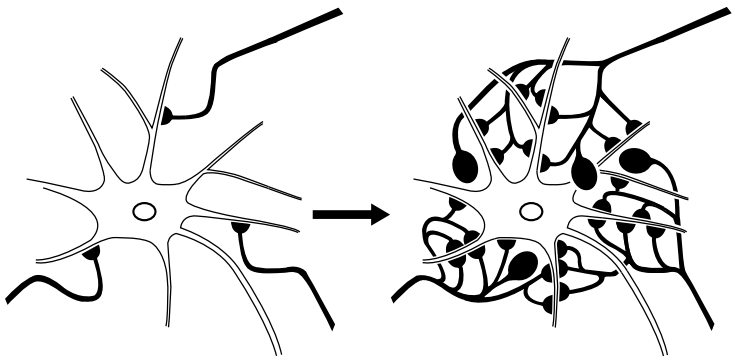
Chapter 5



Estrogen induces axonal outgrowth in the nucleus retroambiguus-lumbosacral motoneuronal pathway in the adult female cat

ABSTRACT

In 1995, VanderHorst and Holstege (J. Comp. Neurol. 359: 457-475) have discovered a new pathway in the cat, which originates from the nucleus retroambiguus (NRA) and terminates in a distinct set of lumbosacral hindlimb, axial and pelvic floor motoneuronal cell groups. The NRA is a compact group of interneurons located laterally in the caudal medulla oblongata. Its projection to lumbosacral motoneurons is thought to represent the final common pathway for male mounting and for female receptive or lordosis behavior. However, females only display lordosis behavior when they are in estrus, which suggests that the NRA-lumbosacral pathway is only active during estrus. This raised the question whether estrogen affects this pathway.
The effect of estrogen on the NRA-lumbosacral projection was studied lightmicroscopically, using wheat germ agglutinin-horseradish peroxidase (WGA-HRP) as a tracer. The rubrospinal pathway served as control. The density of labeled NRA fibers in their target hindlimb motoneuronal cell groups appeared abundant in estrous and very weak in non-estrous cats. Such differences were not found in the rubrospinal pathway. For electronmicroscopical study, the NRA projection to the semimembranosus motoneuronal cell group was selected. In this cell group, an almost ninefold increase of labeled profiles was found in estrous versus non-estrous cats. Moreover, the semimembranosus motoneuronal cell group contained labeled growth cones in estrous, but not in non-estrous cats. These observations demonstrate that estrogen induces axonal outgrowth of NRA fibers to distinct somatic motoneuronal cell groups. The possible mechanisms underlying this outgrowth are discussed.

INTRODUCTION

The nucleus retroambiguus (NRA) is a compact group of interneurons in the lateral tegmentum of the caudal medulla and has been described in humans, cats, rats, hamsters, and birds (Olszewski and Baxter, 1954; Merrill, 1970; Paxinos and Watson, 1986; Holstege 1989; Ellenberger and Feldman, 1990; Wild, 1993; Gerrits et al., in preparation). Anatomically as well as physiologically, the NRA has been shown to be involved in respiration, defecation, vomiting and vocalization (Merrill, 1971, 1974; Feldman, 1986; Fukuda and Fukai, 1986; Miller et al., 1987; Holstege, 1989; Zhang et al., 1992; Miller et al., 1995). It receives projections from respiration related neurons in the brainstem (Feldman, 1986; Smith et al., 1989; Gerrits and Holstege, 1996), and from the midbrain periaqueductal gray (PAG; Holstege, 1989; VanderHorst and Holstege, 1996). The PAG plays a crucial role in the integration of survival behavior, such as defensive and agressive reactions as well as mating (Bandler et al, 1991; Sakuma and Pfaff, 1979a,b; Ogawa et al., 1991).
In the female as well as in the male cat (VanderHorst et al., 1994; VanderHorst and Holstege, 1995, 1996; V. G. J. M. VanderHorst and G. Holstege, unpublished observations) the NRA projects directly to lumbosacral motoneuronal cell groups innervating a distinct set of hindlimb, axial, and pelvic floor muscles. Combined action of this set of muscles in the female does not serve motor activities as stepping, jumping, scratching, running or other daily activities, but underlies aspects of the receptive posture during mating. Such behavior consists of elevation of the lower back (lordosis), rhythmic movements of the hindlimbs (treading), and lateral deviation of the tail (Michael, 1960). This led VanderHorst and Holstege (1995) to postulate that in female cats the NRA-lumbosacral motoneuronal projection forms the final common pathway for lordosis behavior. Electromyographic studies are currently undertaken by this lab to provide evidence for this hypothesis.
Female reproductive behavior is not displayed in the absence of estrogen, whereas in estrous animals it is prominently present (Beach, 1948; Young, 1961; Clark and Mani, 1994). If the NRA-lumbosacral motoneuronal projection represents the final common pathway for lordosis behavior, the question arises whether estrogen has an effect on this pathway. Therefore, the pathway was studied light- and electronmicroscopically in estrous and in non-estrous cats. The results indicate that estrogen induces axonal outgrowth of NRA fibers to their target motoneurons in the lumbosacral cord.

MATERIALS AND METHODS

Ovariohysterectomy and estrogen treatment

NRA series. Anterograde tracing experiments were performed in 17 adult female cats (Table 1; for general surgical and histological procedures, see chapter 1). Eight females were ovariohysterectomized 4-5 weeks prior to the tracer injection. Five of them were estrogen treated for 7 to 10 days prior to the tracing experiment, receiving daily subcutaneous injections of oestradiol benzoate dissolved in oil (Mycofarm, The Netherlands; 20 i.e. or 0.02 mg/kg/day). After 3 days of treatment, they started to display the typical lordosis behavior when tapping the lower back or presenting them to a male cat. Estrogen treatment was continued until to the day of perfusion. The remaining 3 ovariectomized control cats received no estrogen. Progesteron was not administered, because cats are reflex ovulators, in which progesteron levels start to rise only after intromission by the male or intense vaginocervical stimulation (Dawson and Friedgood, 1940).

Of the remaining 9 non-ovariectomized cats, two were in full estrus, i.e. displayed the complete pattern of estrous behavior during 3-4 days before the tracer injection. The other 7 cats showed no signs of estrous behavior in the two weeks before or in the 3 days after the tracer injection.

Red nucleus control series. In four females, the rubrospinal projections in estrous and non-estrous females were studied (Table 1). Three cases were ovariectomized, of which two did and one did not receive estrogen treatment prior to the tracer injections. The remaining natural case did not display estrous behavior in the two weeks before or in the 3 days after the tracer injection.

WGA-HRP injections

NRA series. The distribution of the NRA-lumbosacral neurons in the cat have been described by VanderHorst and Holstege (1995; see also Fig. 1). Since the NRA extends rostrocaudally over a length of 6-7 mm, multiple needle penetrations were necessary to inject wheat germ agglutinin-horseradish peroxidase (WGA-HRP; Sigma, The Netherlands) throughout its rostrocaudal extent. This reduced the possibility that differences in the injection site would result in differences in the NRA-lumbosacral projections between the cases. WGA-HRP was pressure injected via a glass micropipette using a picopump after dorsal approach and exposure of the caudal medulla. Except for cases 2237, 2251, 2256, 2258, 2267, and 2271, the injections were preceded by an ipsilateral C2 hemisection, which interrupted all ipsilaterally descending, non-NRA pathways to the spinal cord (VanderHorst and Holstege, 1995). The hemisections were made by aspiration with a glass pipette.

Red nucleus control series. To rule out the possibility that estrogen has a similar effect on other descending pathways, the rubrospinal tract was chosen as a control pathway. In two cases (2245 and 2310), small WGA-HRP injections were made in the red nucleus, and in two other cases (2362 and 2363) the red nucleus was injected with similar volumes of tracer as used in the NRA injected cases (Table 1). In case 2310, prior to the injection, an ipsilateral C2 hemisection was made.

Lightmicroscopy

The L3-S3 segments were cut on a freezing microtome into 40 μm thick, transverse sections, except for the respective segments of 8 cases (2251, 2256, 2288, 2307, 2308, 2324 , 2337, and 2353), which were cut on a vibratome into 60μm sections. Every fourth section was processed using the tetramethylbenzidine (TMB; Sigma, The Netherlands) procedure according to Mesulam (1982). Sections of the NRA injected cases 2296 and 2299, and 2307 and 2308, and of the red nucleus cases 2362 and 2363 were incubated simultaneously to exclude eventual differences in density of labeling due to the incubation procedure. The density of anterograde labeling in the spinal cord was microscopically examined with a Zeiss Axioskop under combination of polarized light and darkfield condensor. Photomicrographs of representative sections were taken.

Electronmicroscopy

Cases 2251, 2256, 2288, 2307, 2308, 2324, 2337, and 2353 were examined light- as well as electronmicroscopically. The L3-S3 segments of these cases were cut on a vibratome. Every third section was incubated with TMB and ammoniumheptamolybdate overnight (Olucha et al., 1985). The next day they were processed using the slow osmication method for postfixation of Henry et al. (1985). The tissue was stained 'en bloc' in 1% uranylacetate in bidest, and the slabs were dehydrated in graded series of alcohol and embedded in Epon between dimethyldichlorosilane-coated glass-slides (Vinores et al., 1984). A selection was made of those sections containing anterogradely labeled fibers in the semimembranosus motoneuronal cell group. This is a compact cell group which does not overlap with other motoneuronal cell groups, except for its most rostral and most caudal poles (Romanes, 1951; 1964; V. G. J. M. VanderHorst and G. Holstege, unpublished observations), which made it possible to identify it in unstained sections. The selected tissue was cut into ultrathin sections (60 nm) and studied electron microscopically. To determine the density of labeled profiles per area, in each case the labeled profiles were counted in 32 mazes covering the semimembranosus motoneuronal cell group, each maze measuring 10.000μm^2. The symmetry or asymmetry of the synaptic membrane specialization and the content of the labeled profiles was established. In cases 2324 and 2337, the perimeter of labeled profiles and the length of postsynaptic densities was measured.

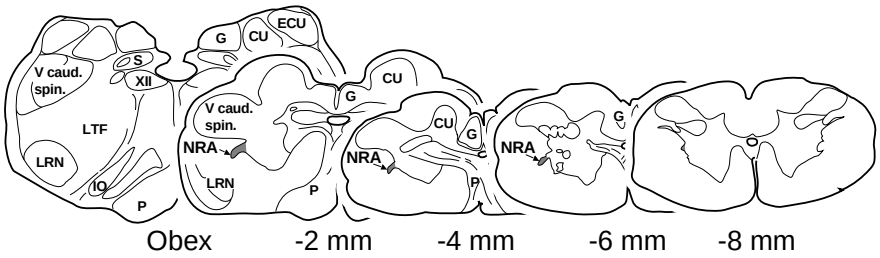


Figure 1 Schematic drawings showing the location of the NRA-lumbosacral neurons in the caudal medulla oblongata. Note that it is most prominant between 2 and 6 mm caudal to the obex where it easily can be recognized as a protrusion of gray matter into the white matter (see also VanderHorst and Holstege, 1995). CU= cuneate nucleus; ECU= external cuneate nucleus; G= gracile nucleus; IO= inferior olive; LRN= lateral reticular nucleus; LTF= lateral tegmental field; NRA= nucleus retroambiguus; P= pyramidal tract; S= solitary complex; V caud. spin.= caudal spinal trigeminus complex; XII= hypoglossal nucleus.

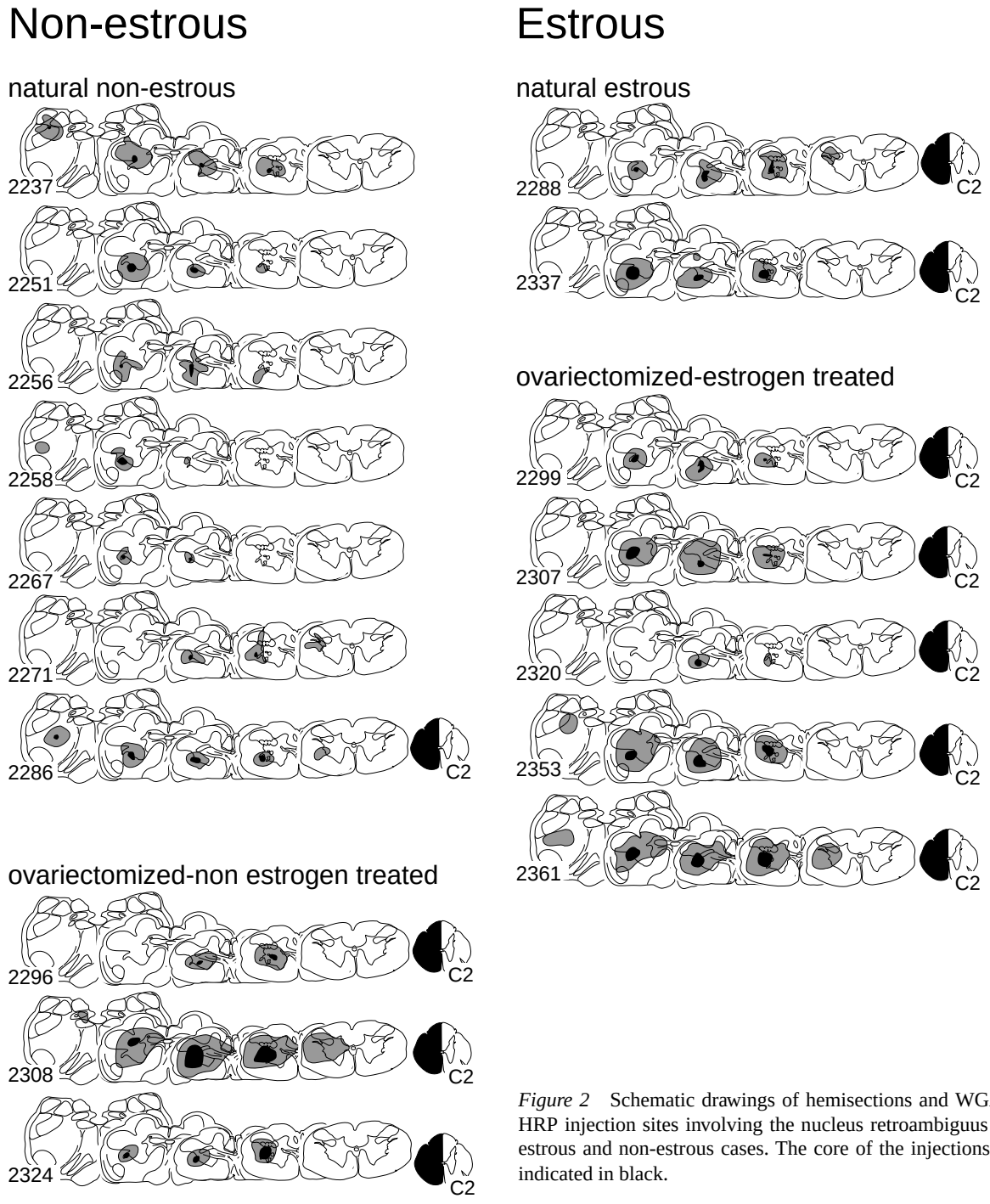


Figure 2 Schematic drawings of hemisections and WGA-HRP injection sites involving the nucleus retroambiguus in estrous and non-estrous cases. The core of the injections is indicated in black.

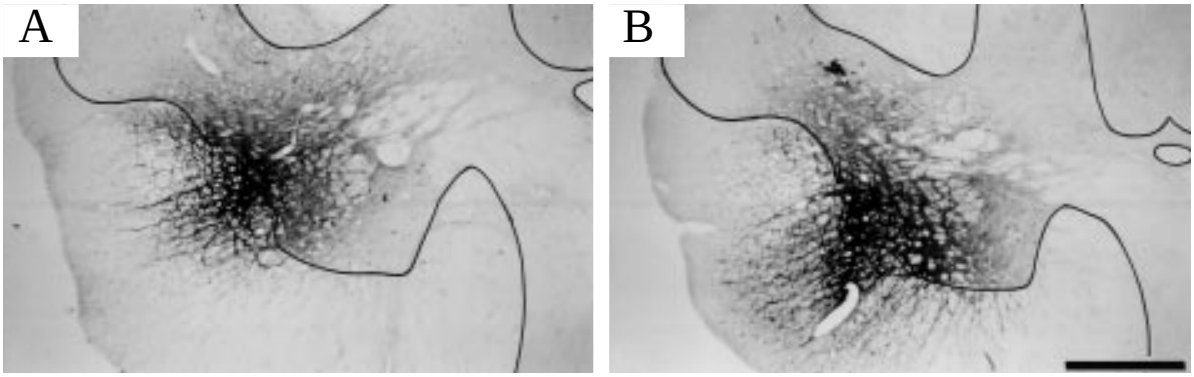


Figure 3 Photomicrographs showing examples of the WGA-HRP injection sites in cases 2324 (left) and 2288 (right). Bar represents 1 mm.

RESULTS

Location and size of the injection sites in the nucleus retroambiguus

In all 17 cases, the injections involved the NRA over a considerable rostrocaudal extent (Fig. 2; Table 1). The hemisections were complete and did not extend across the midline. Examples of the DAB injection sites are shown in cases 2288 and 2324 (Fig. 3).

Non-estrous cases

Lightmicroscopy In the lumbosacral cord of all natural and ovariectomized non-estrous cases (Table I; Fig. 2), the abdominal wall and pelvic floor motor nuclei received a substantial projection (Fig. 4; see Holstege and Kuypers, 1982; Feldman et al., 1985; Holstege and Tan, 1987; Miller et al., 1989; Holstege, 1989). In addition, labeled fibers terminated in the hindlimb motoneuronal cell groups (Fig. 5 left), but they were so sparse that in single sections it was not possible to determine which hindlimb motor nuclei were their main target. However, superposition of 6 consecutive 1:4 sections revealed a distinct projection pattern to the motoneuronal cell groups innervating the muscles iliopsoas, adductor longus, semimembranosus, semitendinosus, biceps femoris anterior and posterior, external anal and urethral sphincter, levator ani, abductor caudae internus, medial longissimus and multifidi (VanderHorst and Holstege, 1995).

Electronmicroscopy In the semimembranosus motoneuronal cell group of cases 2251, 2256, 2308, and 2324, a total number of 25, 9, 12, and 38 labeled terminals were counted per 32 mazes (320.000 µm2), respectively (Table 1). Synapses, which were all asymmetrical, were present in 22% of these profiles. Furthermore, 66% of the labeled trminals contained exclusively spherical vesicles, whereas 13% contained spherical as well as a few dense cored vesicles (Table 2). Of the remaining 22%, the vesicle content could not be identified. Apart

Table 1 Overview of the NRA and red nucleus cases.

NRA	hemi-section	WGA-HRP	number of injections	nl/ injection	total (nl)	Density LM	Density EM
Non-estrous							
natural non-estrous							
2237	-	5%	3	30	90	±	ns
2251	-	5%	2	30	60	±	25
2256	-	5%	5	40	200	±	9
2258	-	5%	2	25	50	±	ns
2267	-	2.5%	2	30	60	±	ns
2271	-	2.5%	3	30	90	±	ns
2286	+	2.5%	5	45	225	±	ns
ovariectomized-non estrogen treated							
2296	+	2.5%	3	50	150	±	ns
2308	+	2.5%	4	50	200	±	12
2324	+	2.5%	3	40	120	+	38
Estrous							
natural estrous							
2288	+	2.5%	4	40	160	++++	281
2337	+	2.5%	4	30	120	+++	157
ovariectomized-estrogen treated							
2299	+	2.5%	3	50	150	+++	ns
2307	+	2.5%	4	50	200	+++	112
2320	+	2.5%	1	30	30	++	ns
2353	+	2.5%	4	40	160	+++	183
2361	+	2.5%	4	30	120	++++	ns
Red nucleus							
Non-estrous							
natural non-estrous							
2245	-	5%	1	15	15	+++++	ns
ovariectomized-non estrogen treated							
2362	-	2.5%	6	25	150	++++++	ns
Estrous							
ovariectomized-estrogen treated							
2310	+	2.5%	2	30	60	+++++	ns
2363	-	2.5%	6	25	150	++++++	ns

The state (estrus or non-estrus), the concentration and volume of tracer injected, and the density of the projections to the lumbosacral cord at the light- and electronmicroscopical level are indicated. ns= not studied; LM= lightmicroscopical level; EM= electronmicroscopical level.

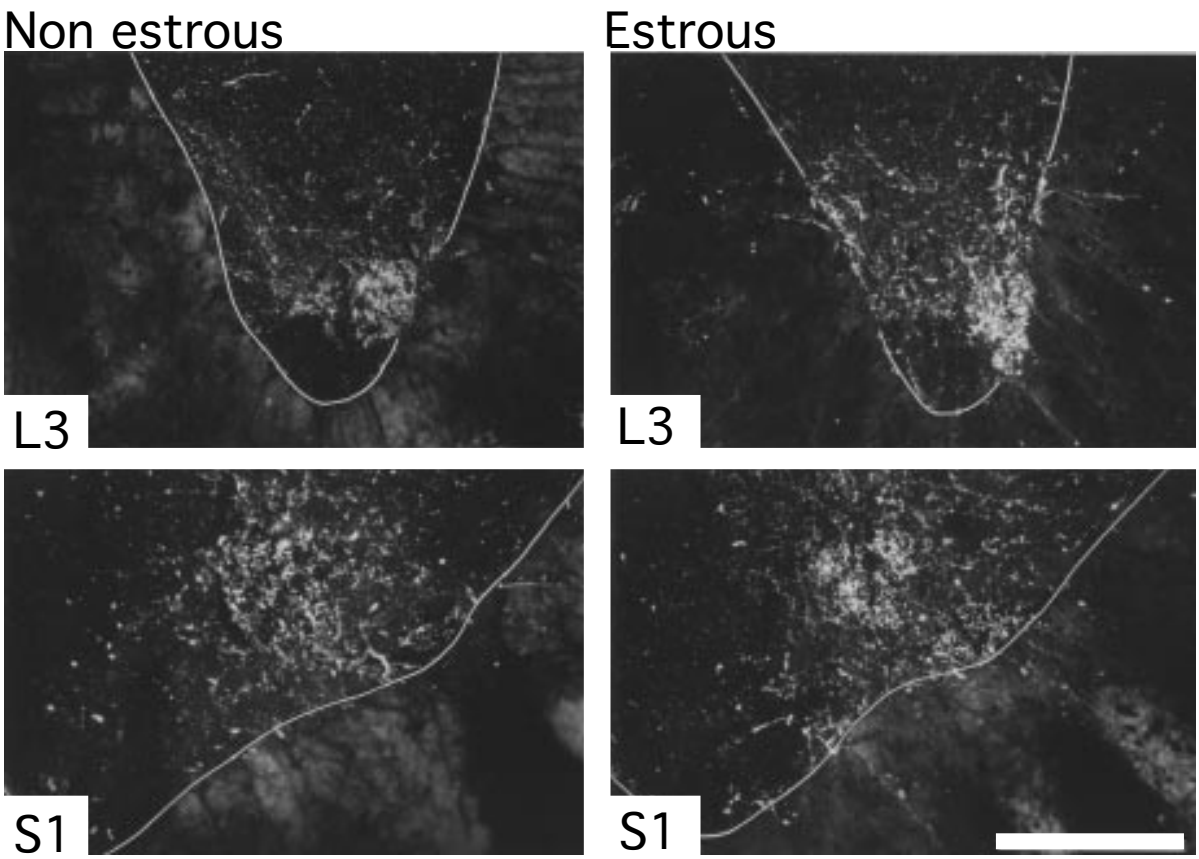


Figure 4 Darkfield-polarized light photomicrographs, showing labeled NRA fibers in the abdominal wall motoneuronal cell groups at the L3 level and in Onuf's nucleus at the S1 level in non-estrous (left; case 2324) and estrous cats (right; case 2288). Note that the NRA projections to the abdominal wall and pelvic floor motor nuclei are slightly denser in estrus than in non-estrus. Bar represents 400 μm.

from spherical and dense cored vesicles, labeled terminals frequently contained some clustered mitochondria (Fig. 6 left). Labeled terminals with flattened or pleiomorphic vesicles were never observed.
In one case (2324), the average perimeter of labeled profiles was determined, and amounted 7.41μm (n=38; ranging from 3.29 to 14.43 μm). On average, 6.9% of it was covered by synaptic junctions. Labeled profiles predominantly contacted dendrites (Fig. 6 left), and only very occasionally neuronal somata.

Estrous cases

Lightmicroscopy In all natural and ovariectomized estrous cases, the NRA-lumbosacral projection was remarkably dense. In sharp contrast to the non-estrous cases, the projections to motoneuronal cell groups innervating the iliopsoas, adductor longus, semimembranosus, semitendinosus, biceps femoris anterior and posterior, and levator ani/abductor caudae internus were very prominent and could easily be discerned in single sections (Fig. 5 right). The differences between estrous and non-estrous cases in respect to the density of projections to the abdominal or pelvic floor motoneuronal cell groups were less apparent. These projections are equally prominent in estrous and in non-estrous cases (Fig. 4).
Between the cases of the estrous group, some differences in density of the NRA-lumbosacral projection were present. In natural estrous case 2288 and in ovariectomized, estrogen treated case 2361, the projections were extremely strong. In case 2320, with a small injection in the NRA, labeled fibers were not as numerous as in the other estrous cases, but still far outnumbered the NRA fibers in the non-estrous cases, including those with large NRA injections (Fig. 2; Table 1).

Electronmicroscopy The number of labeled terminals per 320.000 μm^2 in the semimembranosus motoneuronal cell group of estrous cases 2288, 2307, 2353, and 2337 amounted 281, 112, 183, and 157, respectively (Table 1). The average number of labeled terminals per maze differed significantly between the group of estrous and the group of non-estrous cases (Wilcoxon-Mann-Whitney test; $p<0.025$; Fig. 7). In 14% of the labeled terminals in the 4 estrous cases, synapses were observed, which were all asymmetrical. Of the labeled profiles, 63% contained spherical vesicles and 11% spherical as well as dense cored vesicles. In the remaining 26%, the vesicle content could not be identified (Table 2). No labeled profiles with flattened of pleiomorphic vesicles

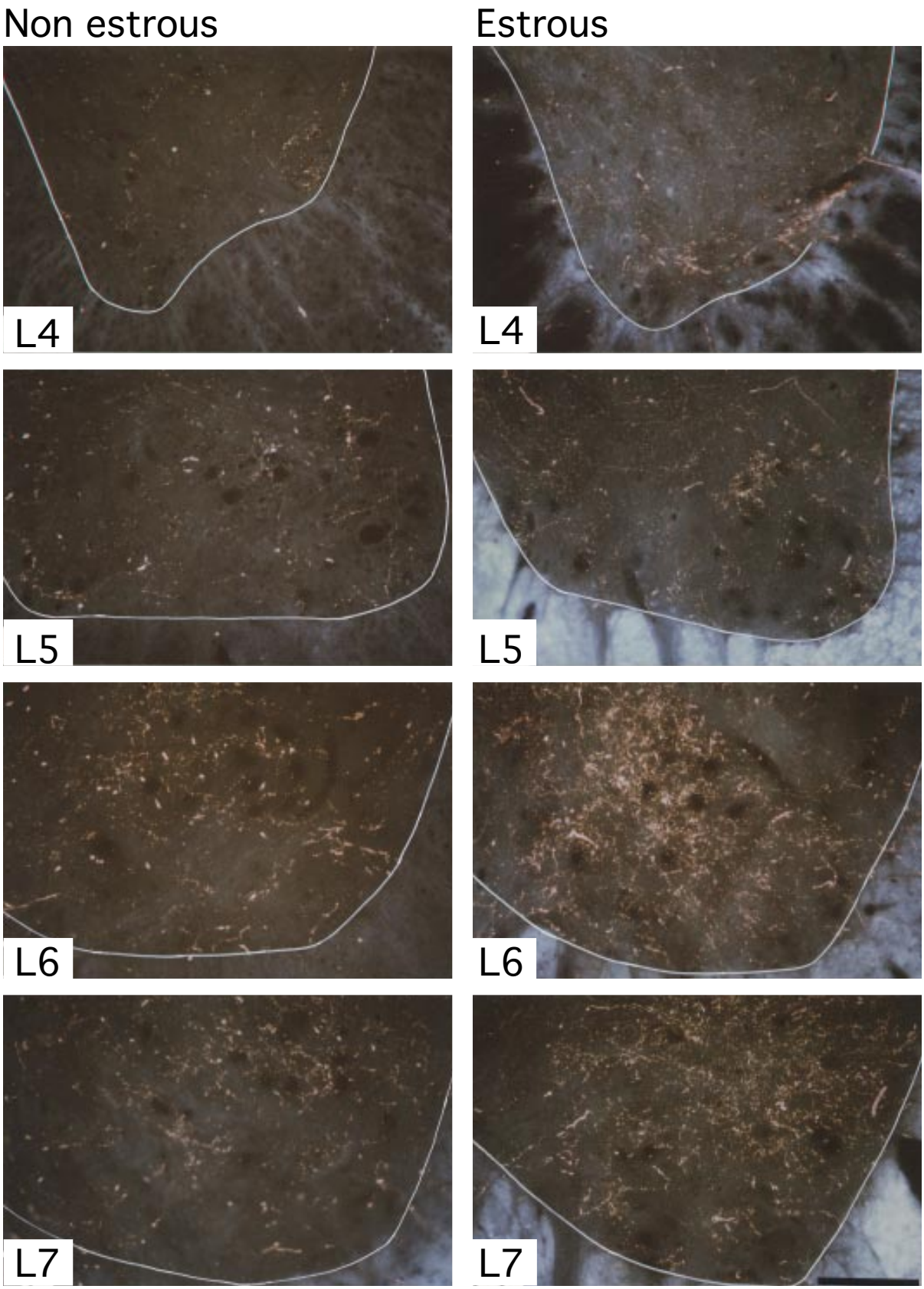


Figure 5 Darkfield-polarized light photomicrographs of labeled NRA fibers in the motoneuronal cell groups of the iliopsoas (L4), adductor longus (L5), semimembranosus (L6), and biceps anterior (L7) in non-estrous case 2324 (left) and estrous case 2288 (right). Note that large difference in the density of NRA fibers between the non-estrous and the estrous case. Bar represents 300 μm.

Table 2 Labeled NRA profiles in the semimembranosus motoneuronal cell group of non-estrous and estrous cases.

	total number of labeled terminals	*labeled terminals with asymmetrical synapses*		*labeled terminals in which no synaps was observed*		*non identifiable labeled terminals*
		spherical vesicles	spherical and dense cored vesicles	spherical vesicles	spherical and dense cored vesicles	
		# (%)	# (%)	# (%)	# (%)	# (%)
Non-estrous (n=4)	84	14 (17)	4 (5)	41 (49)	7 (8)	18 (21)
Estrous (n=4)	732	67 (9)	33 (5)	398 (54)	45 (6)	189 (26)

In each of the cases, a total area of 320.000μm^2 was examined in the same region of the spinal cord.

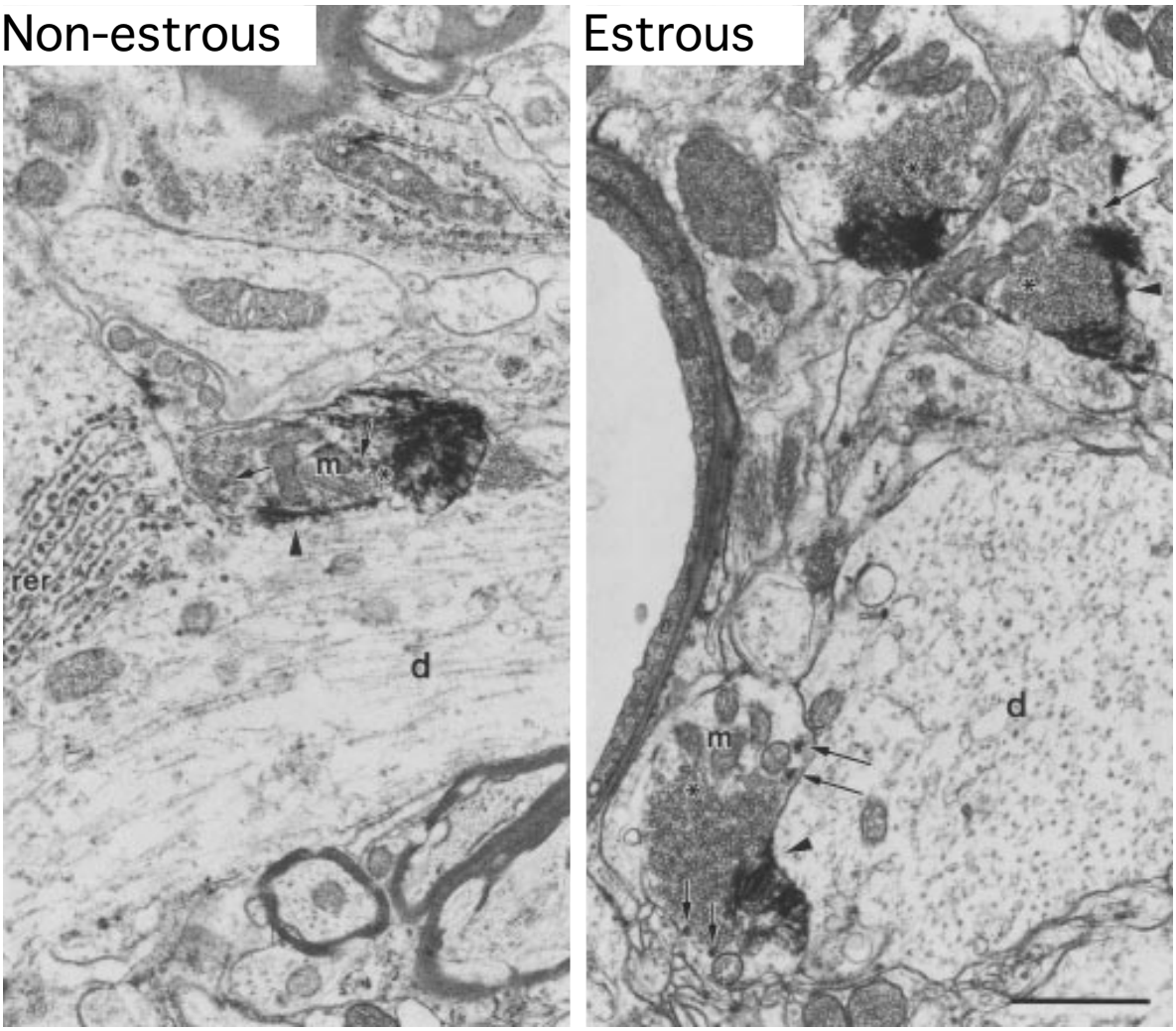


Figure 6 Electronmicrographs showing examples of labeled NRA profiles in the semimembranosus motoneuronal cell group of non-estrous case 2324 (left) and estrous case 2337 (right). In the non-estrous case, a labeled axo-dendritic profile is shown with closely packed spherical vesicles (asterisk), dense-cored vesicles (small arrows), a few mitochondria (m), and an asymmetric synaptic junction (arrowhead). The terminal is located at the initial segment of a dendrite (d), which contains a few cisternae of endoplasmic reticulum with ribosomes (rer). In the estrous case, three large labeled axo-dendritic terminals are present with densely packed spherical vesicles (asterisk), and some dense core (small arrows) and large granulated vesicles (large arrows). Two of them exhibit asymmetrical synaptic membrane specializations (arrowheads). Bar represents 1 μm.

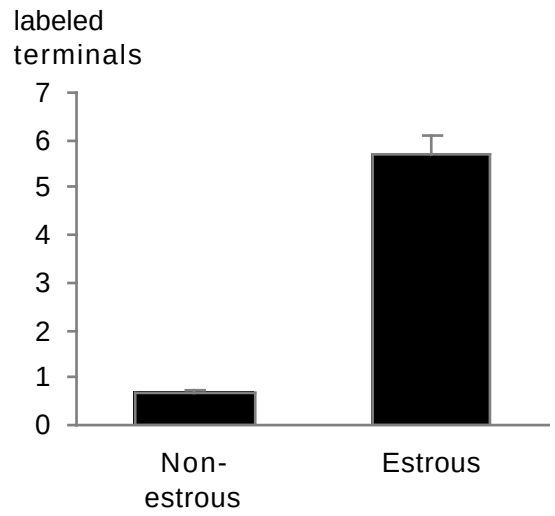


Figure 7 The average number of labeled profiles/10.000μm^2 (± S.E.M.) in the groups of non-estrous and estrous cases. The density of labeled profiles differs significantly ($p<0.025$; Wilcoxon-Mann-Whitney test).

were observed. The labeled terminals mainly contacted dendrites and only very occasionally neuronal somata. In case 2337, the average perimeter of the labeled terminals was 9.49 μm (n=73; ranging from 5.25 to 22.25 μm), which is 1.28 times larger than in the non-estrous case 2324 (7.41μm). In these labeled terminals, synaptic complexes on average formed 5.0% of the perimeter, which is less than in the non-estrous case 2324 (6.9%). In the area under examination (the semimembranosus motoneuronal cell group), the absolute number of labeled profiles displaying synapses was much higher in the estrous cases as compared to non-estrous cases (100 and 18, respectively; see Table 2). In contrast, the percentage of labeled profiles showing one or more synaptic junctions was larger in non-estrous cases than in estrous cases (21% and 14%, respectively).

In all estrous cases, but not in non-estrous cases, very large labeled structures were found which contained large quantities of tightly packed mitochondria (297, 159, 86, and 32 mitochondria per 35.6, 25.4, 12.9, and 8.5μm2), and some agranular reticulum, a few coated, dense core and large granulated vesicles, lysosomes and electron dense particles, and microtubuli (Figs. 8 left and 9). The mitochondria had a smaller diameter and were more elongated than the mitochondria in adjacent structures (Figs. 8 and 9). Similar structures have been described as the proximal or central part of neuronal growth cones in the developing central nervous system (Tennyson, 1970; Yamada et al., 1971; Bunge et al.,

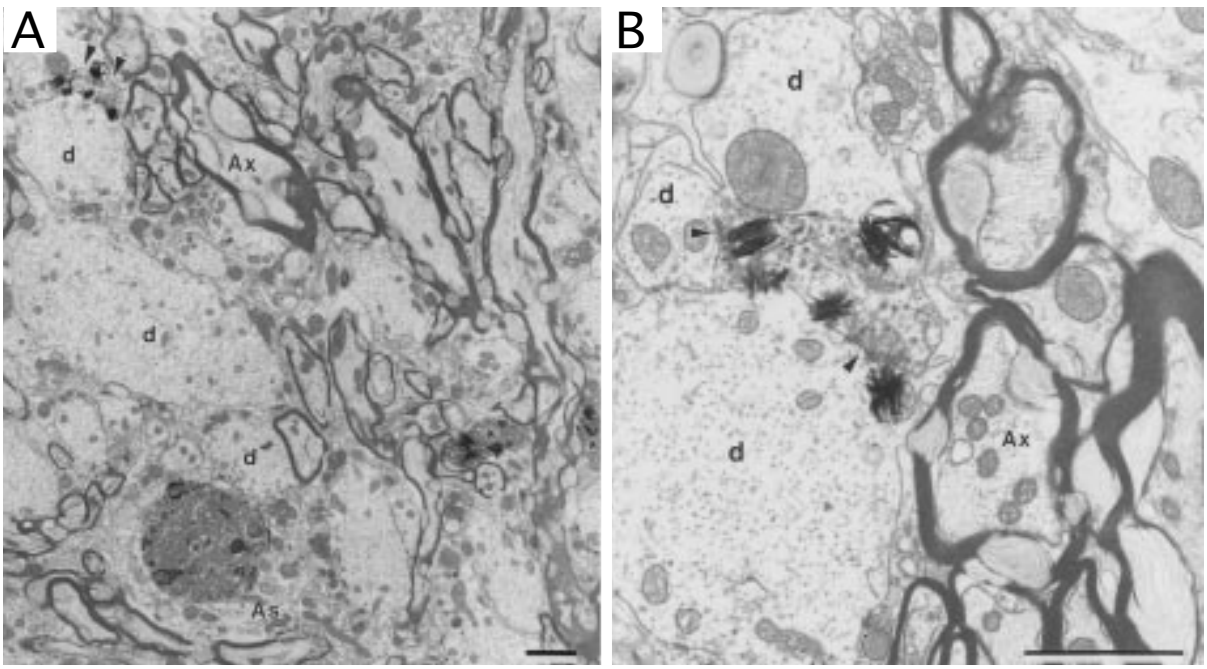


Figure 8 Electronmicrographs of labeled structures in the semimembranosus motoneuronal cell group of case 2337 (natural estrus). A shows one or more axonal growth cones (asterisks in A) parts of an axonal growth cone and a terminal profile (arrowheads). The growth cones contain densely packed mitochiondria, and do not form synaptic contacts. The labeled profile in B is a magnification of the labeled terminal profile in A (one section difference). It is filled with spherical synaptic vesicles, dense core and large granulated vesicles, and forms asymmetrical synaptic junctions with two dendrites (arrowheads); d=dendrite; As=astrocyte; Ax=axon. Bars represent 1 μm.

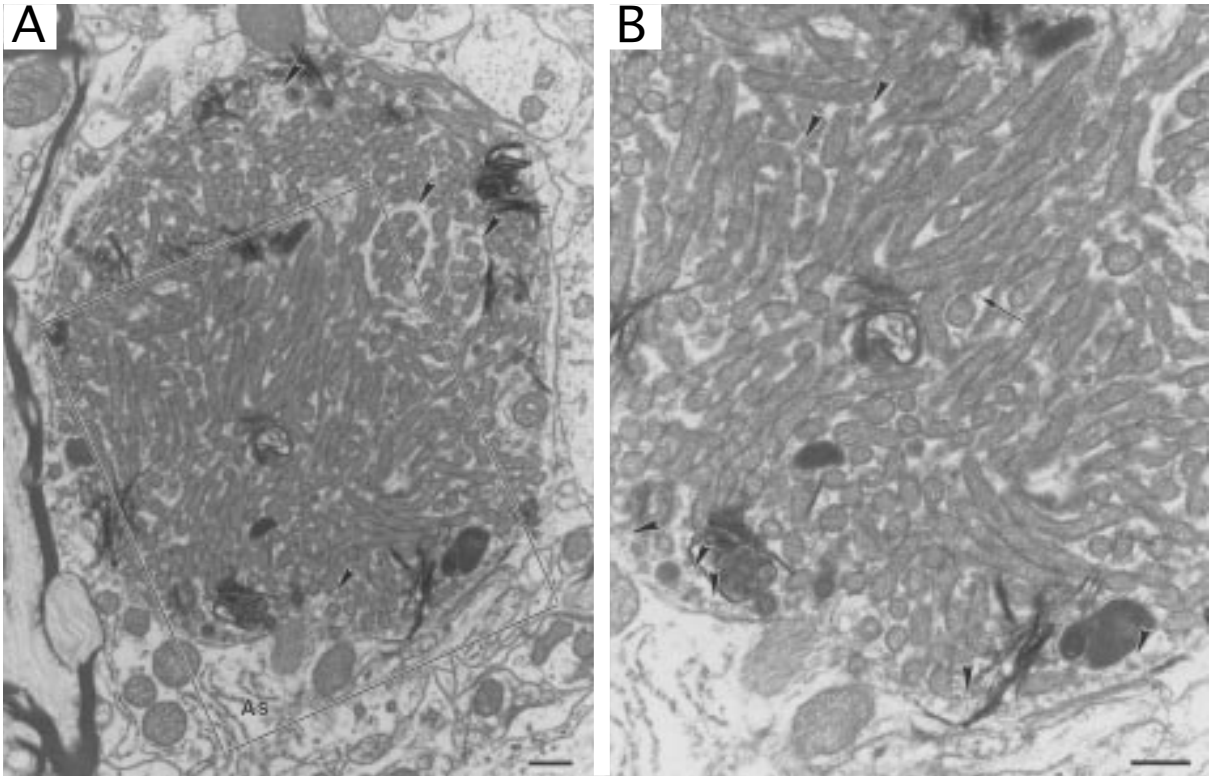


Figure 9 Electronmicrographs representing a labeled growth cone in the semimembranosus motoneuronal cell group of estrous case 2337, for a large part surrounded by astrocytes (As). The mitochondria in the labeled profile have a small diameter, are elongated, and are oriented in or transverse to the plane of the section. Small groups of mitochondria are sequestered within agranular membranes (arrowheads in A). Apart from the mitochondria and smooth membranes, the labeled profile contains a few electron dense bodies, microtubuli (arrow in B), some spherical, dense core, and large granulated vesicles (arrowheads), and an occasional coated vesicle. The majority of the vesicles is located in close proximity to the cytoplasmic membrane. Note that some TMB reaction product appears to be incorporated into the smooth membranes and plasmamembrane. Bars represent 0.5 μm.

1973; Bridgman and Dailey, 1989; Davis et al., 1992), which leads to the conclusion that the large labeled profiles in the present material (4.6% of the labeled profiles in the four estrous cases) represent the proximal parts of growth cones.

Furthermore, labeled, large terminal-like profiles were observed, which contained small diameter elongated mitochondria, cisternae of agranular reticulum, spherical synaptic vesicles, dense cored vesicles, large granulated vesicles, vacuoles, coated vesicles, and a few microtubules and neurofilaments (Figs. 8 and 10). Similar assemblies of organelles have been described in growth cones and immature terminals (Tennyson, 1970; Yamada et al., 1971; Bunge et al., 1973; Vaughn and Sims, 1978; Knyihar-Csillik et al., 1986; Bridgeman and Dailey, 1989; Peters et al., 1991). The presence of large numbers of coated vesicles in the NRA terminals suggests a high level of membrane turnover (Rees et al., 1976) which is thought to be involved in the formation of axonal collaterals (Vaughn and Sims, 1978). At the postsynaptic site, mitochondria, multivesicular bodies, coated vesicles and polyribosomes were found frequently (Fig. 10). Postsynaptically located polyribosomes, which have been described to be particularly prominent during periods of synaps growth, might synthesize proteins that are important for axonal outgrowth or maturation of the synapatic junction (Steward and Falk, 1986, 1991).

In conclusion, many of the labeled NRA profiles in estrous cases contain growth cones and immature terminals, which indicate that outgrowth of NRA axons takes place when the animal is in estrus.

Rubrospinal control series

In 4 cases, WGA-HRP was injected in the red nucleus and surrounding tegmentum (Fig. 11). In all these cases, the density of anterogradely labeled fibers in the intermediate zone of the lumbar enlargement was denser than in the motoneuronal cell groups of NRA injected cases, and was easily visible using brightfield illumination. The two large injections resulted in a slightly stronger projection (non-estrous case 2362 and estrous case 2363) than the smaller red nucleus injections (non-estrous case 2245 and estrous case 2310). However, comparing the estrous with the non-estrous cases with similar injection sites did not reveal any difference in the density of labeling at the lightmicroscopical level.

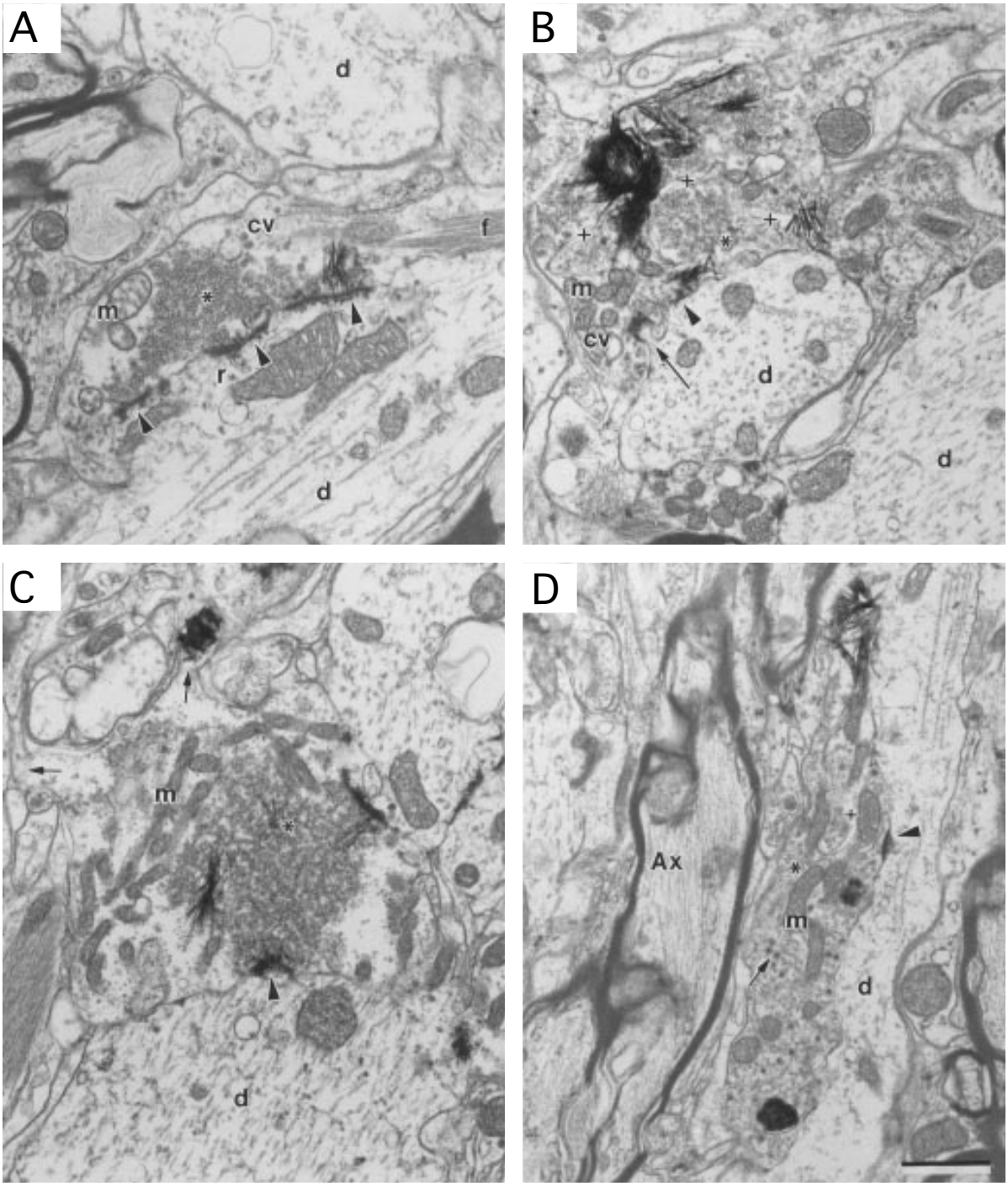


Figure 10 Electronmicrographs of labeled NRA axo-dendritic terminals in the semimembranosus motoneuronal cell group in estrous cases 2353 (A), 2337 (B and C), and 2288 (D).
In A, a labeled terminal is shown which exhibits 3 asymmetrical complexes (arrowheads) with subsynaptic dense bodies. The profile contains a bundle of neurofilaments (f), densely arranged spherical synaptic vesicles (asterisk), a few dense core vesicles, and numerous coated vesicles, some of which seem to originate from double-membrane particles (cv). The matrix of the mitochondria (m) in the labeled profile is not as dense as that of the mitochondria in adjacent profiles. Postsynaptically, large mitochondria (m), cisternae of agranular reticulum, and free ribosomes (r) are present. B shows a large labeled terminal containing large quantities of spherical synaptic vesicles (asterisk), coated vesicles (cv), dense core and large granulated vesicles, cisternae of smooth membranes (+), and a small cluster of mitochondria (m). The terminal forms asymmetrical complexes with a dendrite (arrowhead) and a dendritic spine (arrow). C demonstrates a large labeled terminal with many densely packed spherical vesicles (asterisk) and elongated mitochondria (m) contacting a dendrite (arrowhead). The arrows indicate extensions of the terminal. In D, a labeled profile is present establishing an asymmetrical synaptic contact (arrowhead) with a small dendrite (d). The terminal contains mitochondria (m), sheets of smooth membranes (+), dense core and large granulated vesicles (arrow), spherical vesicles, and numerous small cisternae of agranular reticulum. Bar represents 1 μm.

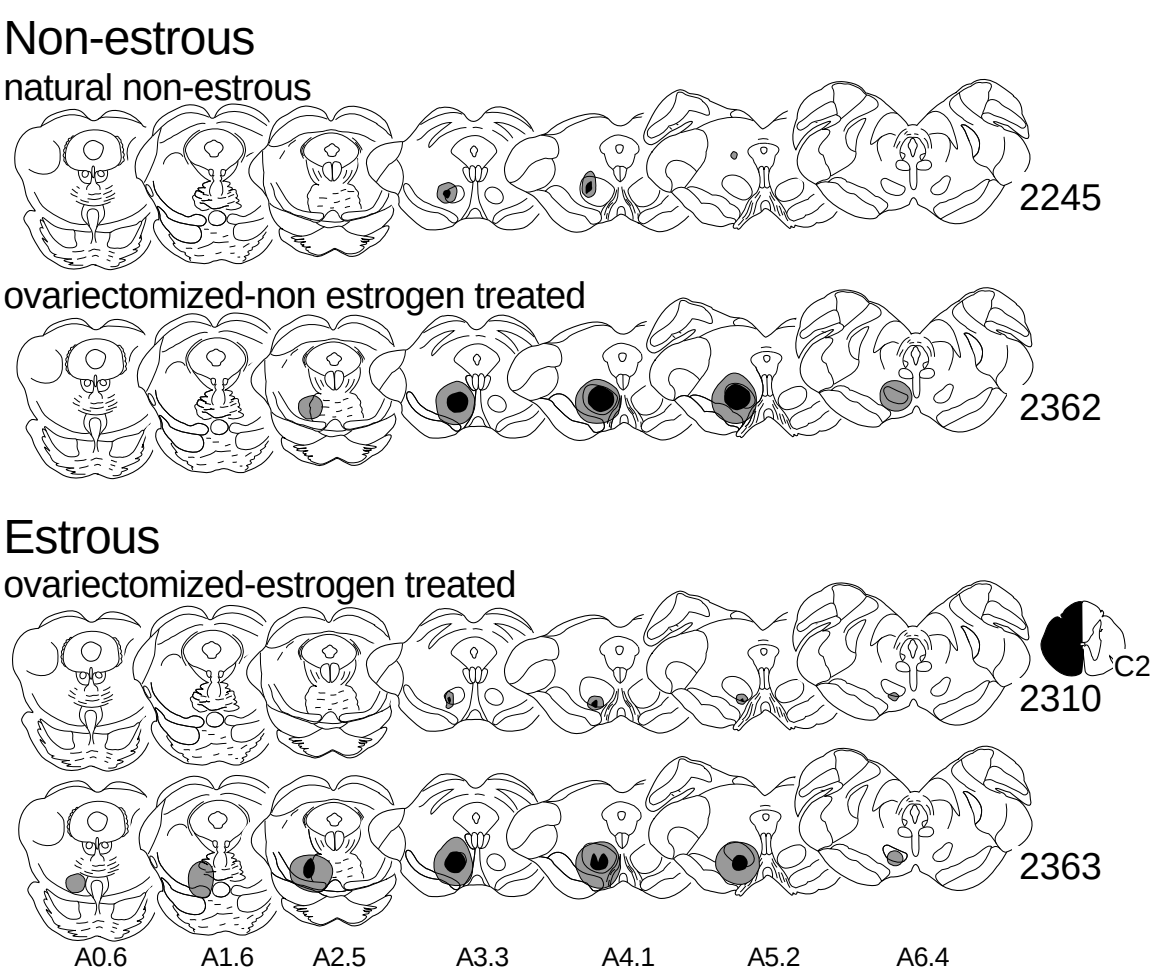


Figure 11 Overview of the WGA-HRP injection sites involving the red nucleus in estrous and non-estrous cases. A0.6 to A6.4 indicate the anterior-posterior coordinates according to the atlas of Berman (1968).

DISCUSSION

Using a new, detailed overview of the spatial location of lumbosacral motoneuronal cell groups (VanderHorst and Holstege, unpublished obser-vations), it could be determined with great precision which of these cell groups received NRA afferents.

The present results demonstrate that the density of the pathway from the NRA to hindlimb motoneuronal cell groups shows significant estrogen related differences in adult female cats. The electronmicroscopical results confirmed the lightmicroscopical observations, demonstrating an almost ninefold increase in the number of NRA profiles in estrous cases. These major differences cannot be fully explained by the small differences in the size of NRA profiles in estrous and non-estrous cases (estrous versus non-estrous= 1.28: 1). The finding of labeled axonal growth cones in the semimembranosus motoneuronal cell group in estrous cats, which were never found in any of the non-estrous cats, demonstrate that the difference in number of labeled profiles is probably based on the formation of new NRA terminals. The finding that the percentage of labeled profiles with one or more synaptic junctions decreases in estrous as compared to non-estrous cases is in line with this hypothesis. Furthermore, electronmicroscopical examination of the semimembranosus cell group in estrous and non-estrous cases showed that the labeled profiles contained mainly spherical and dense cored vesicles and formed asymmetrical synapses, suggesting a mainly excitatory role for the NRA-lumbosacral pathway (see Holstege, 1989). The NRA profiles can be classified as S- or NFs-type (Conradi, 1969; McLaughlin, 1972).

Summarizing, the number of probably excitatory NRA profiles in the semimembranosus motoneuronal cell group increases enormously in estrous cases as compared to non-estrous cases. The presence of labeled growth cones in estrous (natural as well as ovariectomized-estrogen treated), and the absence of such structures in non-estrous cats (natural as well as ovariectomized) suggests that the difference in density in the NRA-lumbosacral projection is based on estrogen-induced outgrowth or sprouting of NRA axons (Fig. 12).

Possible mechanisms underlying sprouting of NRA-axons

The finding that estrogen induces outgrowth of the NRA-lumbosacral pathway leads to the question about which mechanisms are involved in this process. Estrogen has been demonstrated to affect neurons by two different mechanisms: induction of protein synthesis via genomic activation and changing the membrane excitability.

Genomic activation via intracellular estrogen receptors
Estrogen induced protein synthesis is a relatively slow response (hours to days; Pfaff and McEwen, 1983; Clark and Mani, 1994; Pfaff et al., 1994). It is mediated via intracellular receptors, which, when bound to estradiol, activate a specific DNA target (Halachmi et al., 1994). The resulting newly synthesized proteins are transported down the axon (Pfaff and McEwen, 1983; Pfaff et al., 1984; Mobbs et al., 1988), where they are thought to be involved in plastic changes (see Pfaff et al., 1994). Estrogen-related plasticity has been described in cell populations containing intracellular estrogen receptors (Stumpf et al., 1975; Stumpf and Sar, 1976; Pfaff and Keiner, 1973; Rees et al. 1980). These cell groups have a facilitating effect upon lordosis behavior in adult mammals. Examples are the lateral septum (Miyakawa and Arai, 1987), ventrolateral part of the ventromedial hypothalamus (Carrer and Aoki, 1982) and PAG (Chung et al., 1988, 1990). Such plastic changes occur within 24-48 hours, in parallel with the estrous cycle in the rat and hamster (Meisel and Luttrell, 1990; Frankfurt et al., 1990; Frankfurt and McEwen, 1991; Olmos et al., 1989; Langub et al., 1994).

The question is whether a similar mechanism underlies axonal outgrowth in the NRA-lumbosacral pathway. Neither the NRA nor its target motoneuronal cell groups contain estrogen receptors or concentrate estrogen (Stumpf et al., 1975; Stumpf and Sar, 1976; Morrell et al, 1982; Rees et al., 1980; Meijer, VanderHorst and Holstege, unpublished observations). However, in the rat the lateral PAG contains estrogen concentrating neurons some of which project to the caudal medulla oblongata (Corodimas et al., 1990). In the cat, estrogen receptor containing PAG neurons target the NRA (VanderHorst, Meijer, and Holstege, unpublished observations). Other estrogen concentrating structures in the forebrain, such as the ventrolateral part of the ventromedial hypothalamus, medial preoptic area, and amygdala, do not have direct connections with the NRA (Holstege, 1991). Possibly, the estrogen-concentrating neurons in the PAG play a major role in preparing the NRA-lumbosacral pathway for its specific action during lordosis behavior.

Another possibility is that estrogen exerts an effect on the NRA-motoneuronal pathway via the muscles involved (see Haydon and Zoran, 1994 for review). For example, in humans it has been demonstrated that the pelvic floor muscle levator ani, but not the rectus abdominis muscle, contain estrogen receptors (Smith et al., 1990, 1993). This would correspond with the finding that, at least in the cat, the motoneuronal cell groups innervating pelvic floor muscles receive NRA afferents (Holstege and Tan, 1987; VanderHorst and Holstege, 1995), whereas the rectus abdominus motoneurons are not targeted by the NRA (Holstege,

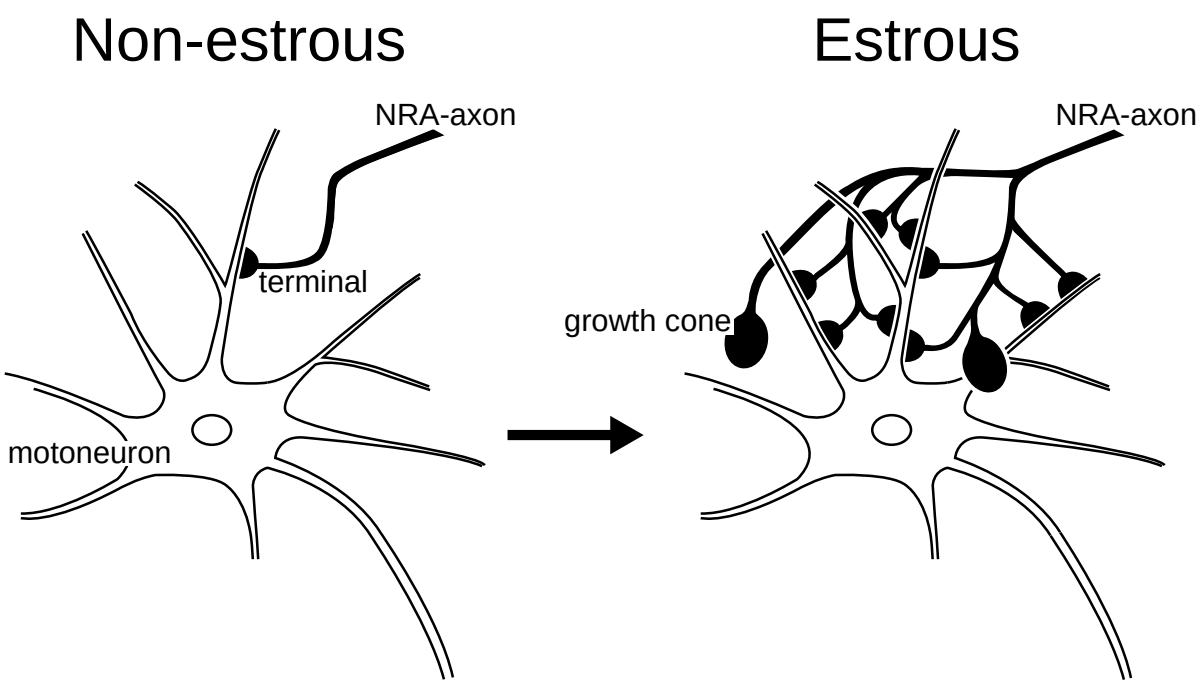


Figure 12 Schematic illustration of the estrogen induced axonal sprouting of NRA fibers to lumbosacral motoneurons. The number of terminals reflects the almost ninefold difference between estrous and non-estrous cases.

1989). Possibly, under high levels of estrogen, estrogen receptor containing muscles send a retrograde signal to their motoneurons, which, in turn, induce outgrowth of NRA axons.

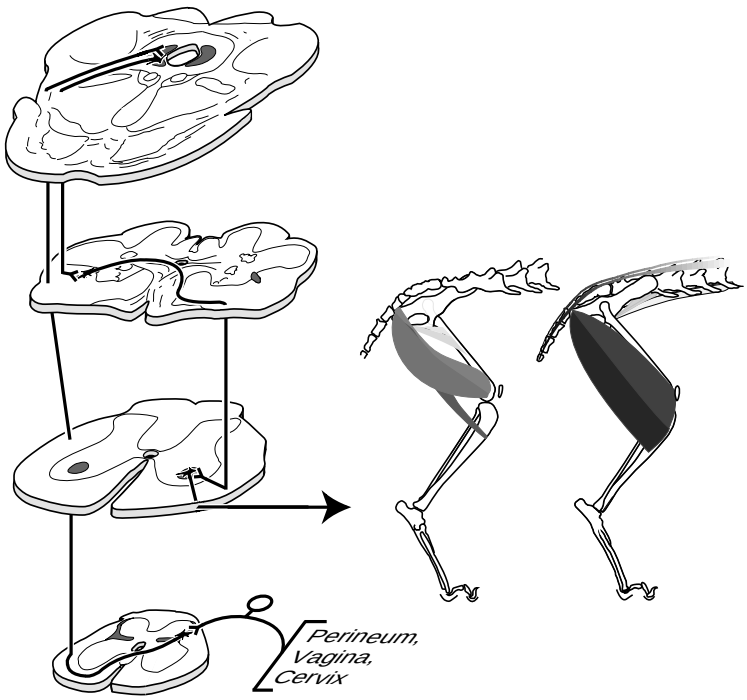
Estrogen effects on membrane excitability Estrogen has also been shown to change the membrane excitability of neurons within minutes (see Alcaraz et al., 1969; Yagi, 1970; Kelly et al., 1977; Dufy et al., 1979; Levesque and Di Paolo, 1988; Schumacher, 1990; Smith, 1994). This effect seems to be mediated via membrane receptors (Schumacher, 1990) which affect calcium channels (Mermelstein et al., 1996). Such rapid effects have been described for numerous cell groups, such as the anterior hypothalamus (Kawakami et al., 1970; Cross and Dyer, 1972; cat, Alcaraz et al., 1969), medial amygdala (Nabekura et al., 1986; Minami et al., 1990), nucleus accumbens (Thompson and Moss, 1994), hippocampus (Wong and Moss, 1991, 1992), and cerebellum (Smith et al., 1987, 1989). Whether this mechanism plays a role in the NRA axonal outgrowth is not known.

Functional implications of estrogen induced axonal sprouting in the NRA-lumbosacral pathway

Estrogen has been shown to induce growth or plasticity in mid- and forebrain structures (Meisel and Luttrell, 1990; Frankfurt et al., 1990; Frankfurt and McEwen, 1991; Olmos et al., 1989; Langub et al., 1994; Miyakawa and Arai, 1987; Carrer and Aoki, 1982; Chung et al., 1988, 1990), as well as in non-neuronal structures as the uterus (Burrows, 1949; Reynolds, 1951; Clark and Mani, 1994 for review). However, the present study is the first to show that estrogen induces the outgrowth of a long brainstem-spinal motor pathway, which is thought to represent the final common pathway for lordosis. The effect of estrogen on this pathway seems to be rather specific, because no estrogen-related differences could be detected in the rubrospinal motor tract.

These findings are in line with the notion that estrogen or other sex steroids are necessary for activation of the reproductive neural circuitry, which appears to be latently present in non-estrous animals.

General discussion



General discussion

In this thesis, a new concept is put forward for the basic circuitry (from spinal cord to brainstem to spinal cord) for mating behavior in the cat. Relevant stimuli are conveyed directly from the lumbosacral cord to the PAG, where this information is integrated with input from the forebrain (Fig. 1). Via interneurons in the NRA, the PAG activates a specific set of lumbosacral motoneurons. The muscles innervated by these motoneuronal cell groups are involved in the receptive posture in females and in the mounting posture in males.

However, posture is only one of the components of reproductive behavior. Female receptive behavior for example is accompanied by immobility, changes in nociception, cardiovascular responses, vocalization, and pupil dilatation. The present chapter discusses the basic neural circuitry for receptive and mounting behavior in the context of these other behavioral components, estrogen effects, and species differences. In addition, the role of the NRA as relay for the emotional motor system is discussed.

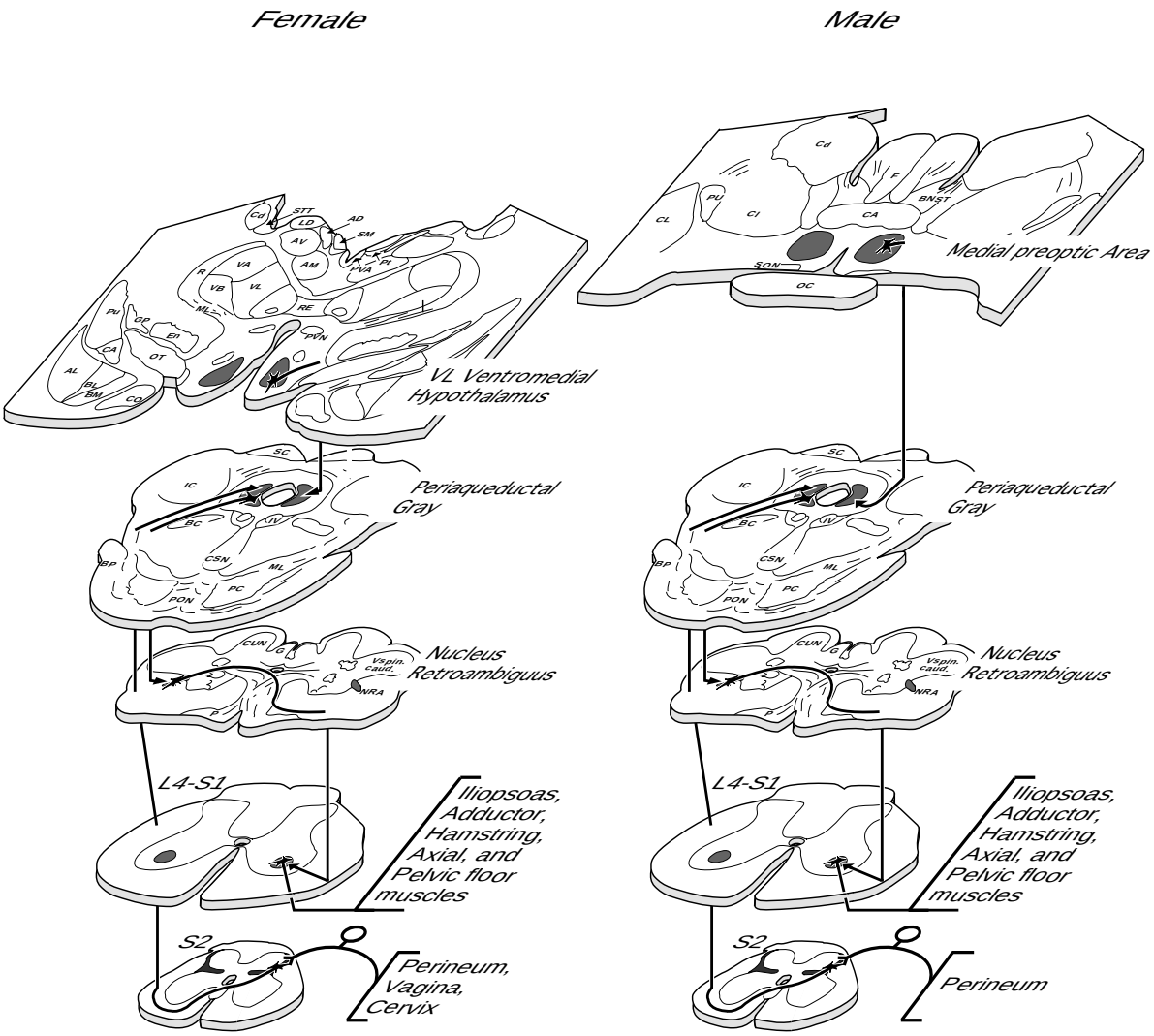


Figure 1 Schematic representation of the basic neural circuitry for female receptive (left) and male mounting posture (rigth).

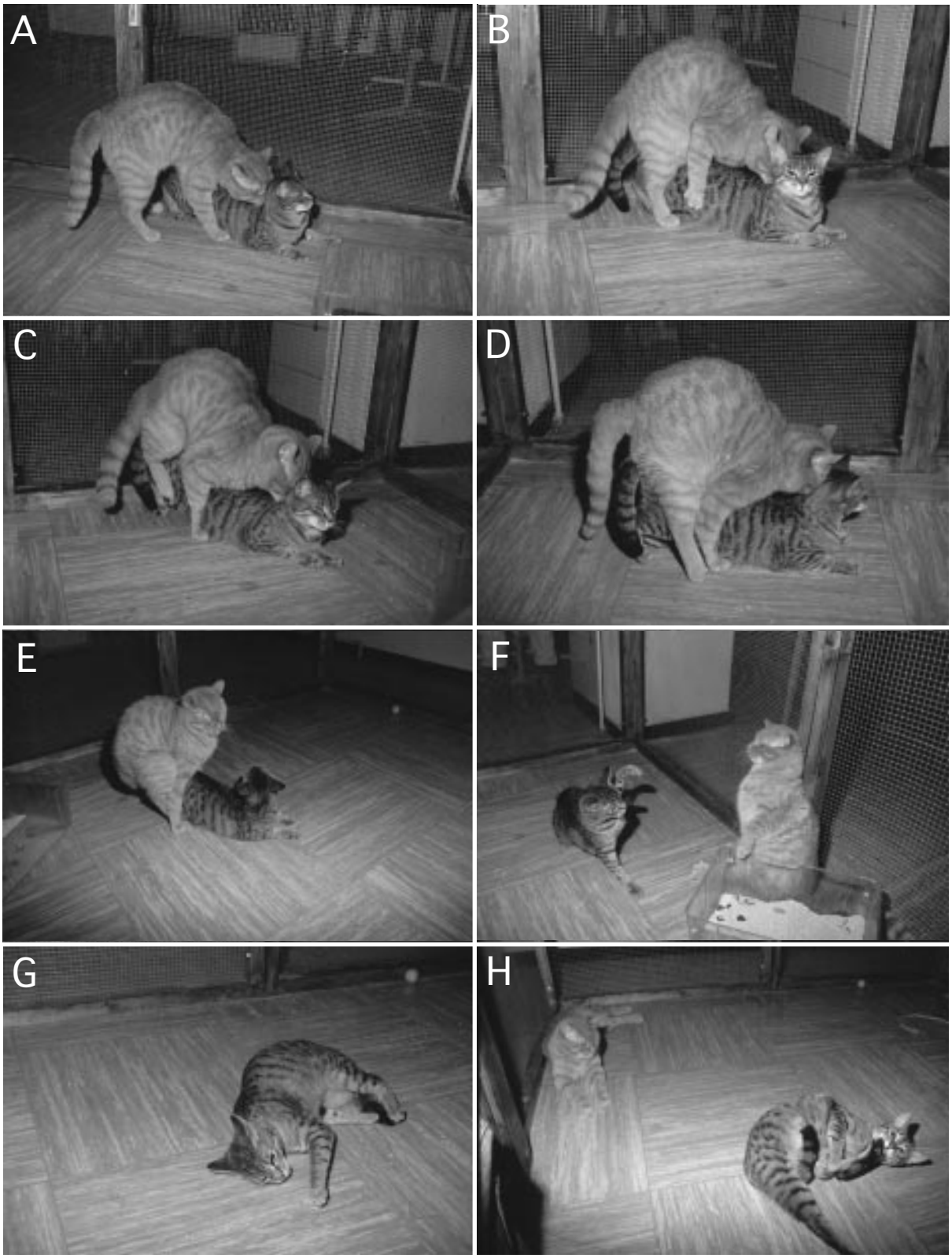


Figure 2 Photomicrographs of mating cats. The male shows the neck grip (A), palpation of the flanks (B), and treading (C and D). The female displays receptive behavior, which consists of a crouching posture (A to E), lateral deviation of the tail (C and D), treading (D), and elevation of the lower back (A toD). After ejaculation of the male, the female emits the "estrous cry", becomes very aggressive (E and F), and starts rolling (G) and licking (H). This behavior is called the "after-reaction".

Female and male sexual behavior in the cat

During mating, male and female cats display their own repertoire of motor behaviors (Michael, 1961; Whalen, 1963). In order to attract a male, an (pro)estrous female displays so called proceptive behavior, which consists of calling (vocalization), and increased locomotor activity. Olfactory stimuli from the vaginal fluid of the estrous female trigger male mounting behavior, which starts with the neck grip (Fig. 2A). The neck grip immobilizes the female, and allows the male to mount the female. Mounting is characterized by grasping with the forelimbs (forelimb rubbing Fig. 2B), hindlimb stepping (Fig. 2C), arching of the back, stamping on the female cat's rump (palpation of the flanks), and finally pelvic thrusting. These sensory stimuli induce the receptive posture in the female (Fig. 2A to D). In the female cat the full receptive posture consists of crouching (forelegs collapsed), lowering of the head, perineal elevation (lordosis), tail deviation, and treading (rhythmical movements of the hindlimbs). This posture, also called lordosis, enables intromission by the male. Receptive-mounting behavior usually takes several minutes and is continued until ejaculation. At that moment, the female emits the "estrous cry" (a distinctive form of vocalization) and turns very aggressively towards the male (Fig. 2E and F). She starts rubbing and rolling over the floor, and vigorously licks the perineal area, the tip of the tail and the toes. This so-called "after reaction" lasts for 15-20 minutes, after which the male is allowed to mount again. Matings can continue for many hours.

The basic neural circuitry for female receptive behavior

I. Different components of receptive behavior and their relevant spinal input (Fig. 3a and e)

Receptive posture Receptive behavior is initiated easily in freely moving animals by applying tactile stimuli to the skin of the flanks, posterior rump, tailbase, and perineum (e.g., for the rat: Kow et al., 1979; for the cat: Michael, 1961). Going from the flanks to the perineum, these stimuli have an increasing effect on the strength of the lordosis response. The strongest response can be elicited by vaginocervical stimulation. Under natural conditions, all these stimuli are applied by the mounting male. The lordosis relevant sensory input is conveyed from the perineal skin, vagina and cervix to the lumbosacral cord mainly through the pelvic and pudendal nerves (Morgan et al., 1981; Ueyama et al. 1984 ; Szechtman et al., 1985; Thor et al., 1989; Gomora et al., 1994).

Vocalization Althoug vocalization can be considered as an independant emotionally driven motor activity, the "estrous cry" is very distinctive. It is a typical component of receptive behavior and marks the transition between receptive behavior and the after reaction. The "estrous cry" can only be evoked after vigorously stimulating the vaginocervix. Normally, this is achieved during and after ejaculation by the penis of the male cat, which bears multiple little spines.

Analgesia, blood pressure changes, and pupil dilatation Apart from the lordotic posture and immobility, receptive behavior is also characterized by less overt reactions such as analgesia (Komisaruk and Wallman, 1977; Catelli et al., 1987; Gomora et al., 1994), blood pressure changes (Catelli et al., 1987), pupil dilatation (Szechtman et al., 1985), and endocrine responses. All these reactions can be evoked by natural or artificial vaginocervical stimulation (Szechtman et al., 1985; Catelli et al., 1987; Gomora et al., 1994). Mounting alone has been shown to be far less potent (Komisaruk and Wallman, 1977; Szechtman et al., 1985; Gomora et al., 1994). The responses elicited by vaginocervical stimulation are mediated via the pelvic nerve, because bilateral transection of this nerve (and hypogastric nerve; see Cunningham et al., 1991) abolishes them (Szechtman et al., 1985; Gomora et al., 1994).

Immobility Immobility of the female cat can be evoked by vaginocervical stimulation (Naggar and Komisaruk, 1977 in the rat). However, before mounting, the male cat licks or even bites the female in the neck, which also immobilizes her (Whalen, 1961). Afferents conveying this sensory input from skin and muscles of the neck terminate in the upper cervical cord (Abrahams et al., 1984).

Endocrine responses Vigorous stimulation of vagina and cervix during intromission by the male also triggers endocrine responses, such as the release of prolactin and oxytocin from the pituitary (for review see Komisaruk and Steinman, 1986). Prolactin in turn stimulates the release of the ovarian hormone progesteron, which prepares the uterus for implantation. Oxytocin provokes uterine contractions, during parturition known as the Ferguson reflex. In reflexive ovulators, such as cat and rabbit, coital stimulation induces ovulation by the pulsatile release of luteinizing hormone-releasing hormone (LH-RH, also called gonadotrophin releasing hormone or GnRH), followed by the release of luteinizing hormone (LH) (Concannon et al., 1980; Banks and Stabenfeldt, 1982; for review see Komisaruk and Steinman, 1986). This mechanism can be abolished by transection of the pelvic nerves (Wildt et al., 1980; Cunningham et al., 1992).

II. Spino-PAG pathways (Fig. 3b)

Manual vaginocervical stimulation of estrogen primed cats with a hemisection in the C2 segment of the spinal cord results in "hemi-lordosis" (unpublished observations), which is characterized by extension of the lower

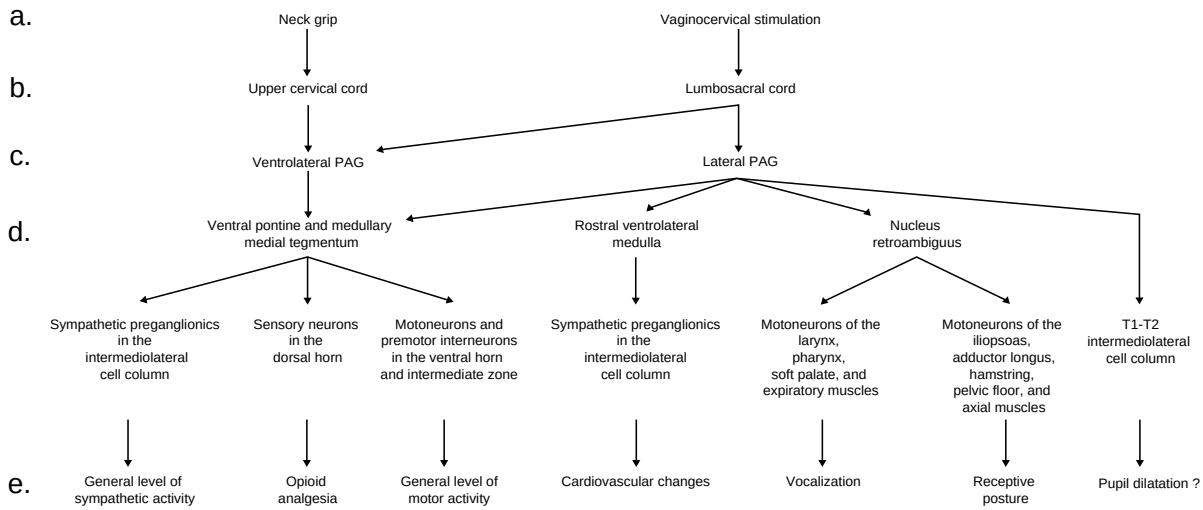


Figure 3 Scheme showing the different components of receptive behavior and the pathways which are possibly involved.

back and treading of only the hindlimb located contralateral to the hemisection. These findings indicate that receptive behavior is not mediated at the level of the spinal cord, but requires supraspinal control. Thus, in order to evoke receptive behavior, the information from the genital area and the neck has to be conveyed to supraspinal levels.

Without forebrain structures (after precollicular decerebration), receptive behavior can still be elicited by somatosensory and vaginocervical stimulation in ovariectomized cats and rats (Bard and Macht, 1958; Rose and Flynn, 1993). Vaginocervical stimulation selectively increases the metabolic activity in the rat PAG (Allen et al., 1981), and activates the intermediate-early gene c-fos in this area (Pfaus et al., 1993; Tetel et al., 1993). Lesions in the PAG abolish receptive behavior, which demonstrate that this midbrain structure is essential for an integrated pattern of this behavior (Sakuma and Pfaff, 79b).

Physiological studies have shown that the PAG receives lordosis relevant somatosensory input (Pfaff and Schwartz-Giblin, 1988 for review). Theoretically, this information reaches the PAG indirectly or directly. A possible indirect pathway involves the nucleus tractus solitarius (NTS) which responds to stimulation of the cervix, uterus, and vagina (Hubscher and Berkley, 1994; 1995). Via projections from the NTS to the PAG, this information might reach the PAG (Herbert and Saper, 1992). The NTS receives vaginocervical input via a spinal cord pathway, and not via the vagal nerve (Hubscher and Berkley, 1995). However, after tracer injections into the sacral cord, only a very sparse projection is present in the NTS (unpublished observations), suggesting a minor or no role for this indirect pathway in receptive behavior.

Anatomical tracing studies in the rat, cat, and monkey have demonstrated that the PAG receives direct projections from the spinal cord (Menetrey et al., 1982; Wiberg and Blomqvist, 1984; Yezierski, 1988; VanderHorst and Holstege, 1992; VanderHorst et al., in press; chapter 1; Mouton, VanderHorst and Holstege, in prep.). Spino-PAG neurons are not evenly distributed throughout the spinal cord. Especially the upper cervical and sacral segments send a major projection to the PAG (VanderHorst and Holstege, 1992; Mouton, VanderHorst and Holstege, in prep.). Spino-PAG neurons in the upper cervical segments might very well convey stimuli from the "neck-grip" to the PAG. At sacral levels, the location of spino-PAG neurons matches precisely the location of pelvic primary afferents (VanderHorst and Holstege, 1992; VanderHorst et al., in press; Chapter 1), which implicates that these convey information relevant for receptive behavior to the PAG.

III. Integration in the PAG (Fig. 3c)

After the information important for receptive behavior has converged in the PAG, different behavioral components have to be combined into an integrated behavioral pattern. Especially the lateral and the ventrolateral PAG are of importance for this task.

The lateral PAG

Receptive posture The sacral-PAG neurons that convey pelvic visceral information, have axons terminating medially in the lateral part of the caudal PAG (VanderHorst et al., in press; chapter 1). In estrogen primed rats, lesions in the same area of the PAG have been shown to decrease lordosis posture (Sakuma and Pfaff, 79b), whereas stimulation in the lateral and dorsal PAG facilitates lordosis (Sakuma and Pfaff, 1979a).

Estrous cry, aggressive behavior, blood pressure changes, and pupil dilatation The lateral PAG is not only important for receptive posture. Stimulation in the lateral part of the lateral PAG evokes aggressive and defensive behavior, which is accompanied by hyper-

tension, vocalization, tachycardia, pupil dilatation, and non-opioid analgesia (Sakuma and Pfaff, 1979a; Bandler and Depaulis, 1991). Therefore, it is very well possible that the lateral PAG mediates the increase in blood pressure, pupil dilatation, as well as the estrous cry and the sudden hostile reaction displayed by the female after mating. The vigorous stimuli from the spined penis of the male, which are conveyed from the sacral cord to the lateral PAG, are likely to initiate the sudden change from immobile and receptive behavior to aggressive reactions, including the "estrous cry".

The ventrolateral PAG

Immobility and opioid dependant analgesia In the most caudal PAG, sacral cord neurons terminate in the ventrolateral area (VanderHorst et al., in press; Chapter 1). Stimulation in this region has been shown to elicit quiescence and hyporeactivity, accompanied by hypotension, bradycardia, and opiod dependant analgesia (Bandler et al., 1991; Bandler and Depaulis, 1991; Carrive and Bandler, 1991; Lovick, 1993). The ventrolateral PAG also receives input from the upper cervical cord (Keay and Bandler, 1992; Mouton et al., in prep.). Therefore, this part of the PAG might be involved in the immobility evoked by the neck grip and vaginocervical stimuli.
Since analgesia evoked by vaginocervical or uterocervical stimulation is opioid dependant (Watkins et al., 1984; Hill, 1980; Gintzler and Komisaruk, 1991), this component also might be integrated in the ventrolateral PAG.

Endocrine responses Coital stimulation triggers the release of prolactin, oxytocin, and luteinizing hormone from the pituitary. A direct sacral cord-hypothalamic pathway (Burstein et al., 1990; Katter et al., 1991) might be involved in these mechanisms. However, since this tract is only very small in the cat (Katter et al., 1991), it is more likely that an indirect sacral cord-hypothalamic pathway is responsible for these endocrine responses. Possibly the PAG plays the role of intermediate structure. Moreover, it has been shown that LH-RH-containing axons of hypothalamic neurons are present in the ventrolateral PAG, immediately adjacent to the aqueduct (Liposits and Sétáló, 1980; Shivers et al., 1983; Veening et al., 1991). Injection of LH-RH in the PAG facilitates lordosis behavior (Riskind and Moss, 1979; Sakuma and Pfaff, 1980; 1983; Sirinathsinghji, 1984; Pfaff et al., 1994 for review). This facilitating effect of LH-RH could also be observed in hypophysectomized animals (Pfaff, 1973), which indicates that it is not caused by the release of LH from the pituitary.

IV. PAG-spinal pathways involved in receptive behavior (Fig. 3d)

The PAG controls specific motor patterns by means of distinct pathways belonging to the lateral emotional motor system, whereas it has a global, modulatory effect

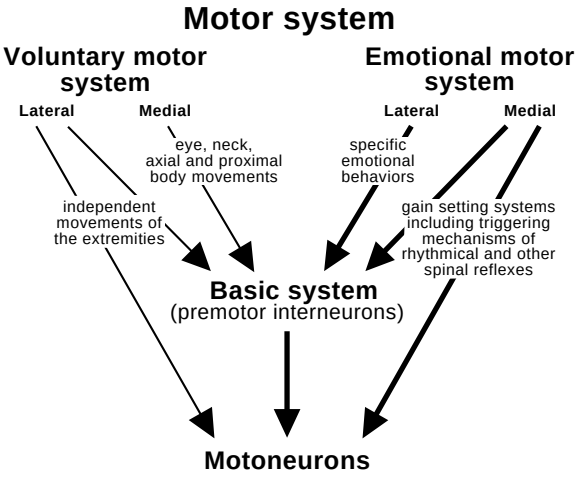


Figure 4 Schenatic overview of the three subdivisions of the motor system (from Holstege, 1996).

on motor output via the diffuse projections of the medial emotional motor system (see Fig. 4). Both the specific and diffuse pathways play a role in reproductive behavior.

Specific pathways belonging to the lateral emotional motor system

Direct PAG-spinal projections The shortest way for the PAG to reach the spinal cord is by means of a direct pathway. Recently, direct PAG-spinal projections have been described from the ventrolateral and lateral intermediate PAG to the medial ventral horn mainly at the level of the cervical and upper thoracic cord (Mouton and Holstege, 1994). The function of this projection is not known, but it might play a role in the control of axial muscles during threat display (arching of the back; Mouton and Holstege, 1994). Since only a limited number of fibers continue into the lumbosacral cord, containing the motoneurons of the muscles involved in receptive behavior, it is not likely that the reproductive posture is mediated by this direct PAG-spinal projection. However, this pathway might reach lordosis motoneurons via propriospinal interneurons in the cervical cord.
Another direct PAG-spinal projection originates from the lateral PAG and projects directly to preganglionic sympathetic motoneurons in the intermediolateral cell column of the upper thoracic cord (Holstege, 1988; see Fig. 3). This pathway has been hypothesized to mediate pupil dilatation (Holstege, 1988).

PAG-NRA-motoneuronal projections The NRA receives a specific and direct projection from the lateral, the dorsal, and the caudal ventrolateral PAG (Holstege, 1989; VanderHorst and Holstege 1996; introduction) and in turn projects directly to a selective set of axial, hindlimb, and pelvic floor motoneuronal cell groups (VanderHorst and Holstege, 1995; in press; chapters 3

and 4; see Fig. 1). Taking into account the function of the muscles innervated by these motoneurons, the NRA pathway cannot be involved in jumping and running, but is suitable for receptive behavior (chapter 3). In conclusion, the anatomical findings indicate that the NRA forms a relay between the PAG and a distinctive set of lumbosacral motoneuronal cellgroups. Currently, electromyographical studies are underway to test the hypothesis that this pathway indeed controls receptive behavior.
The NRA also projects to motoneurons innervating the larynx, pharynx, soft palate, and expiratory muscles (Holstege, 1989). Thus, the PAG-NRA pathway also controls vocalization (Holstege, 1989; Zhang et al., 1992; Davis et al., 1996), which includes "calling" and the "estrous cry".

The rostral ventrolateral medulla The lateral PAG is known to send excitatory projections to the subretrofacial nucleus, also called rostral ventrolateral medulla (Lovick et al., 1984; Lovick, 1985; Li and Lovick, 1985), which in turn projects to sympathetic preganglionics in the intermediolateral cell collumn of the thoracic and upper lumbar cord (Loewy and McKellar, 1981; Loewy et al, 1981). The sympathoexcitatory neurons in the rostral ventrolateral medulla determine the level of sympathetic outflow to different vascular beds (Lovick, 1987; Dampney and McAllen, 1988). Thus, the lateral PAG-rostral ventrolateral medulla-spinal cord pathway might mediate the cardiovascular changes evoked by vaginocervical stimulation (Catelli et al., 1987).

Diffuse pathways belonging to the medial emotional motor system

Ventral pontine and medullary medial tegmentum Both the ventrolateral and lateral PAG reach motoneurons in the spinal cord indirectly by way of projections to the ventral medullary medial tegmentum (Sakuma and Pfaff, 1980; Lovick, 1993; Lovick, 1996). In contrast to the specific NRA-spinal projections, the pathway from the ventral medullary medial tegmentum, including the caudal raphe nuclei, terminates diffusely in the ventral horn. This pathway, therefore, may play a role during reproduction, not by reflexively activating specific sets of motoneurons (Sakuma and Pfaff, 1980), but by modulating their general excitability via the release of glutamate (extracellularly), serotonin, norepinephrine and associated neuropeptides (White et al., 1996; Holstege, 1996; Lovick, 1996).
This ventral medullary medial tegmentum-spinal pathway might also play a role in an increase in rhythmical (non-goal directed) motor activities (see Holstege, 1996), such as running, rolling, and rubbing during proceptive behavior and the after reaction. However, the PAG itself does not seem to trigger these latter responses, since precollicular decerebration has been demonstrated to abolish them (Bard and Macht, 1958).
In addition, the ventrolateral PAG affects the spinal dorsal horn and intermediate zone, via the pontomedullary raphe magnus and adjacent tegmentum. The projections to the dorsal horn are well known to be involved in opioid dependant analgesia (Oliveras et al., 1974;1975; Fields and Basbaum, 1978). Similar to the modulatory effect of the ventral medullary medial tegmentum on motoneurons in the ventral horn, neurons in the raphe magnus and adjacent tegmentum modulate nociceptive transmission in the dorsal horn (for review see Mason and Leung, 1996). Thus, ventrolateral PAG-pontomedullary tegmentum-dorsal horn pathway might mediate the opioid dependant analgesia induced by vaginocervical stimulation.

Effect of estrogen on the basic neural circuitry for receptive behavior

Mating behavior is dependant on sex steroids. The female is able to display her repertoire only when she is in estrus, i.e. when high levels of estrogen are present and ovulation is ready to take place (Fig. 5). Estrogen is mainly produced by the ovaries, from where it reaches the entire body via the bloodstream.
In the CNS, estrogen induces protein synthesis in estrogen concentrating cell groups (see DeBold and Malsbury, 1983; Meisel and Pfaff, 1984; 1985; McEwen, 1988), but it can also change the excitability of membranes of neurons in general (for review see Smith, 1994). Estrogen concentrating cells are most most abundant in forebrain areas. Examples of estrogen concentrating neuronal cell groups in the forebrain are the medial and central nuclei of the amygdala, the medial division of the bed nucleus of the stria terminalis, the medial preoptic area, anterior hypothalamus, and ventromedial hypothalamus (VMH) (in the rat: Pfaff and Keiner, 1973; Stumpf et al., 1975; in the mouse: Stumpf and Sar, 1975; in the cat: Rees et al., 1980; in the monkey: Keefer and Stumpf, 1975). These structures are all involved in the various aspects of sexual behavior (Pfaus et al., 1993; Tetel et al., 1993), and all project to the PAG (see Saper et al., 1976; Saper et al., 1976; Hopkins and Holstege, 1978; Holstege , 1987b; Veening et al., 1991; Shipley et al., 1991). Thus, via these pathways estrogen might affect the basic neural circuitry for receptive behavior.
Since receptive behavior can also be evoked without input from the forebrain (Bard and Macht, 1958; Rose and Flynn, 1993), this chapter focusses on the effects of estrogen on the motor and sensory pathways of the basic circuitry itself. First the ventromedial hypothalamus is discussed, because of its close relation receptive behavior in general.

The ventromedial hypothalamus Especially the ventrolateral part of the VMH is well known for its role in reproductive behavior. Stimulation and lesions in the

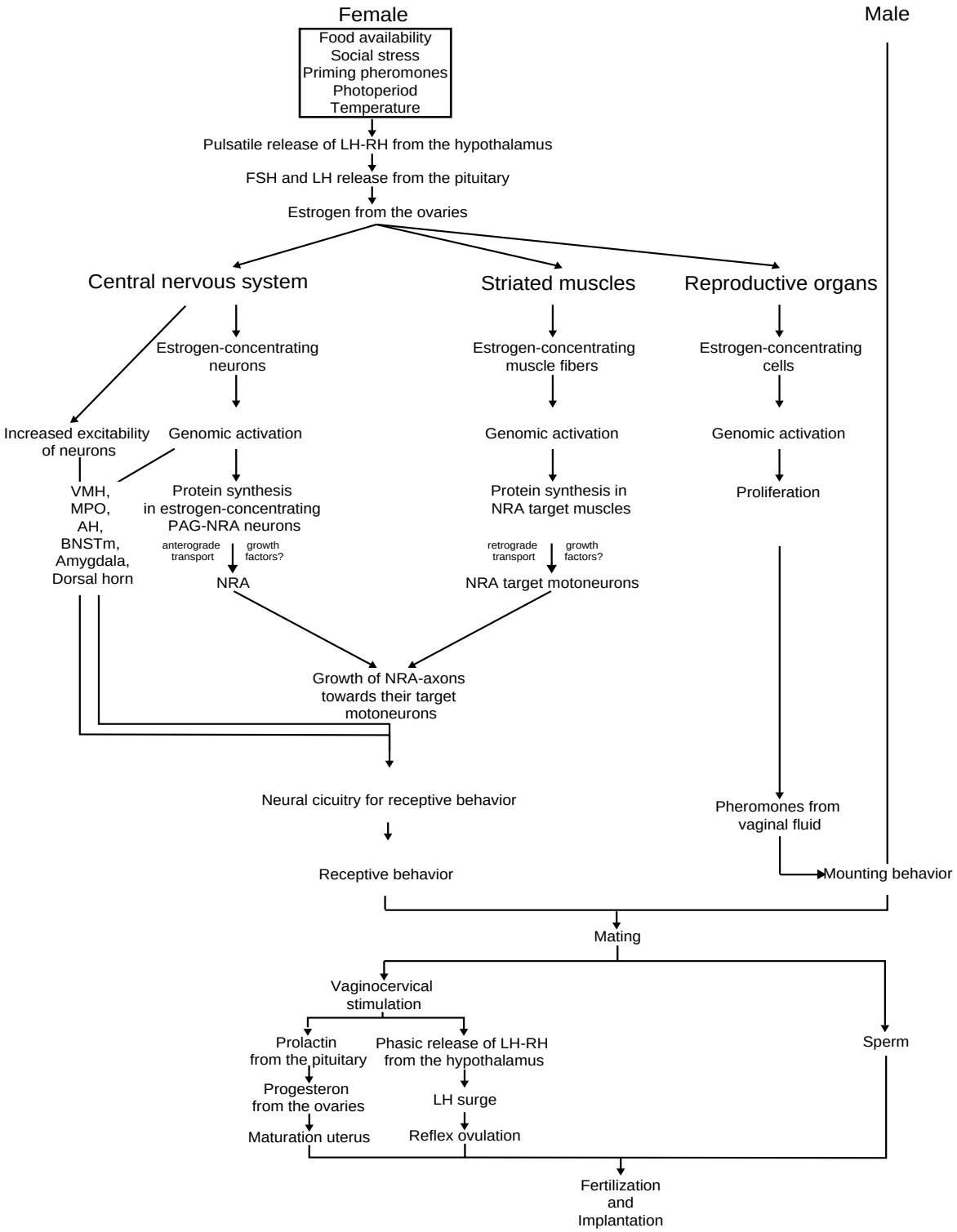


Figure 5 Schematic overview of a concept for the mechanisms necessary for succesful mating.

VMH respectively facilitate and suppress lordosis (Pfaff and Sakuma, 1979a,b for the rat; Mathews and Edwards, 1977; Malsbury et al., 1977 for the hamster; Leedy and Hart, 1985 for the cat). However, lesions in the VMH do not abolish PAG-facilitated lordosis in estrogen primed rats (Pfaff and Sakuma, 1979b), but lesions in the PAG render VMH stimulation unsufficient for evoking receptive behavior (Pfaff and Sakuma, 1979a). Apparently, the VMH is important for estrogen priming, whereas the PAG is the final integrator for receptive behavior, in the same way as it is for vocalization (Davis et al., 1996).

The question remains how the estrogen sensitive VMH acts on PAG neurons. VMH neurons specifically project

to the medial part of the lateral caudal PAG (Canteras et al., 1994). Estrogen affects this pathway by decreasing the stimulation threshold of VMH-PAG neurons (Sakuma and Pfaff, 1982), and by inducing protein synthesis (see DeBold and Malsbury, 1983; Meisel and Pfaff, 1984; 198; Yahr and Ulibarri, 1986; McEwen, 1988) and transport along the axons (Meyerson, 1982; Pfaff et al., 1984). This latter mechanism is consistent with the finding that inhibition of protein synthesis blocks the effect of estrogen on reproductive behavior (Meyerson, 1982)

Motor pathways

Periaqueductal gray Estrogen not only increases the sensory responsivity of hypothalamic neurons (Alcaraz et al., 1969; Beyer et al., 1971), but also of mesencephalic neurons (Beyer et al., 1971). For example, female sex steroids strongly facilitate unit responsiveness to lordosis relevant tactile stimulation (Rose and Bieber, 1984), whereas implants of estradiol in the PAG in hamsters facilitates vocalization and receptive behavior (Floody et al., 1986).

The PAG is the most caudal structure in the brain that contains large quantities of estrogen concentrating neurons (in the rat: Pfaff and Keiner, 1973; Stumpf et al., 1975; in the mouse: Stumpf and Sar, 1975; in the cat: Rees et al., 1980; in the monkey: Keefer and Stumpf, 1975). In the cat, they are mainly located in the lateral PAG and adjacent tegmentum, and to lesser extent in the dorsal PAG (Rees et al., 1980). Thus, apart from an indirect facilitating effect of estrogen via the VMH, the PAG itself is also estrogen sensitive. PAG-estrogen concentrating neurons do not only exert a local effect, but some of them project to the region of the NTS (Corodimas and Morrell, 1990 in the rat) or more precisely to the NRA (unpublished observations of Meijer, VanderHorst and Holstege in the cat). The mechanisms by which estrogen affects the PAG-NRA pathway might be similar to its effect on the VMH-PAG projection, i.e. estrogen decreases the stimulation threshold of the PAG-NRA neurons, and induces protein synthesis and transport along the PAG-NRA axons.

The ventral medullary medial tegmentum At the level of the medulla, estrogen affects the responsivity of neurons in the ventral medullary medial tegmentum (Kow and Pfaff, 1982). This effect might play a role in proceptive behavior and the afterreaction, which behaviors are characterized by a high level of motor activity. Since the ventral medullary medial tegmentum does not contain estrogen concentrating neurons, the changes in responsivity might be the result of an effect of estrogen on the excitability of neuronal membranes in general (Smith, 1994). A more likely explanation is that this area is affected indirectly, because estrogen also increases the excitability of the numerous PAG cells that project to the ventral medullary medial tegmentum (Sakuma and Pfaff, 1980b).

The nucleus retroambiguus According to Rose and Sutin, estrogen also increases the responsivity of "medullary neurons located in the medial lateral reticular nuclei and the region extending dorsomedially to the nucleus ambiguus". These neurons responded at short latency to PAG stimulation (Rose and Sutin, 1973) and represent in all likelihood NRA neurons. Since neurons in this area do not contain estrogen receptors (unpublished observations by Meijer, VanderHorst and Holstege), these changes might be mediated indirectly by the projection from estrogen concentrating cells in the PAG to the NRA (cat; unpublished observations by Meijer, VanderHorst and Holstege).

Recently, it has been shown that estrogen induces outgrowth of the pathway from the NRA to the lumbosacral cord, involved in the receptive posture (VanderHorst and Holstege, see chapter 5). Thus, apart from an effect on the responsivity of NRA neurons, estrogen positive PAG-NRA projections might also play a role in the sprouting reaction of the NRA-lumbosacral terminal fibers.

Motoneurons and muscles Since outgrowth of the NRA-lumbosacral axons is directed only to the NRA target motoneuronal cell groups, it is also possible that these motoneurons attract the NRA fibers. Motoneuronal cell groups themselves do not concentrate estrogen (in the rat: Pfaff and Keiner, 1973; Stumpf et al., 1975; Morrell et al., 1982; in the mouse: Stumpf and Sar, 1975; in the cat: Rees et al., 1980; in the monkey: Keefer and Stumpf, 1975), but some muscles such as the levator ani muscle do (Smith et al., 1990; 1993 in human). In bovine calfs, estrogen receptor concentrations in neck, shoulder and hindlimb muscles did not differ between males and females, but in males the concentration in abdominal wall muscles was lower (Sauerwein and Meyer, 1989). In rats, no estrogen receptors have been found in the quadriceps (Michel and Baulieu, 1980), which is not targeted by the NRA. These findings indicate that estrogen receptors in muscles might be selectively distributed. Possibly, outgrowth of NRA fibers to distinct sets of motoneurons is mediated via a retrograde signal, induced by estrogen, from estrogen concentrating muscle fibers to their motoneurons. By means of growth factors, these motoneurons in turn might attract afferent fibers, such as the NRA fibers. This mechanism will only work if the NRA target muscles, in contrast to other muscles, contain estrogen receptors. This possibility is presently under investigation.

Sensory pathways

Sensory peripheral pathways Regarding sensory stimuli relevant for recepetive behavior, the genital sensory field in female rats has been shown to become larger by estrogen administration (Komisaruk et al., 1972), and the response sensitivity of pelvic afferent fibers is larger on the day of proestrus (Robbins et al., 1992; however see Kow and Pfaff, 1979). This effect might be mediated

by estrogen receptors in the dorsal root ganglion (Sohrabji et al., 1994).

Spinal sensory neurons Extracellular recording has demonstrated that estrogen does not significantly affect the responses of lumbosacral units to lordosis relevant sensory input (Kow et al., 1980). However, laminae I and V throughout the spinal cord contain numerous estrogen concentrating (Morrell et al., 1982), or estrogen receptor containing neurons (Amandusson et al., 1995; in the rat; Meijer, VanderHorst, and Holstege, unpublished observations in the cat). These spinal neurons are sensitive for estrogen and must exert an effect, either at spinal or at supraspinal levels. Possibly, that some of these estrogen receptor containing neurons convey lordosis relevant information to the PAG. This option is currently studied.

The basic neural cicuitry for male mounting behavior

Male reproductive behavior is evoked primarily by olfactory or vomeronasal input (Meisel and Sachs, 1994 for review). Somatosensory stimuli from the penis, which reach the lumbosacral cord via the pudendal nerve, are important for intromission and ejaculation, but play a minor role in the mounting posture (see Lucio et al., 1994). Lesion studies have revealed several important areas in the forebrain which play a role in male reproductive behavior. All these areas are involved in the processing of olfactory input. Bilateral lesions of the olfactory bulbs, affecting both the main and accessory olfactory systems, or the corticomedial nucleus of the amygdala eliminates copulation in rodents (Meisel and Sachs, 1994; Giantonia et al., 1970). However, lesions of the olfactory system do not have such effects in cats (Aronson and Cooper, 1974), dogs (Hart and Haugen, 1972), or the rhesus monkey (Goldfoot et al., 1978). In the medial preoptic area, electrolytic or neurotoxic lesions in cats and rats (e.g. Heimer and Larsson, 1966; Hart et al., 1973; Kelley and Pfaff, 1978; van de Pol and Dis, 1979; Hart and Leedy, 1983; Meisel and Sachs, 1994) have been shown to affect or disrupt male sexual behavior, whereas electrical stimulation facilitates copulatory behavior (rat: Malsbury, 1971; van Dis and Larsson, 1971; opossum: Roberts et al., 1967). The MPO appears not only to be involved mounting, but also in other aspects of male mating behavior such as mating vocalization (in Mongolian gerbils; Holman et al., 1991 ; in mice: Nyby et al., 1992) and urine marking (in cats: Hart and Voith, 1978; in mice: Nyby et al., 1992).

It is not known which structures are involved in mounting behavior at the level of the midbrain. Since the MPO is known to send a major projection to the PAG (Saper et al., 1978; Holstege, 1987), the PAG might be involved. In this concept, the PAG in turn activates the somatic motoneurons of the muscles involved in the mounting posture and vocalization via a relay in the NRA (chapter 4). For urine marking, the M-region in the pons forms the relay between PAG and/or MPO and bladder motoneurons (Holstege et al., 1986; Blok and Holstege, 1996).

Adult male cats have constant levels of sex steroids and are able to mate at any time with an estrous female. This indicates that the neural circuitry for mating behavior in the male cat is not subjected to changes. Preliminary results of VanderHorst and Holstege show that there exist no significant differences in number of labeled NRA-lumbosacral terminals between castrated and non-castrated adult males. The strength of the NRA projection in the castrated and non-castrated males was twofold higher than in non-estrous females. These results are in line with common knowledge that castration of adult male house cats is not effective in abolishing urine spraying and mounting behavior. Possibly, after castration of male cats at a younger age, the strength of the NRA-lumbosacral pathway in these males is less that in males that have been exposed to male sex steroids, and is similar to that in non-estrous females. This option is currently under investigation.

Species differences

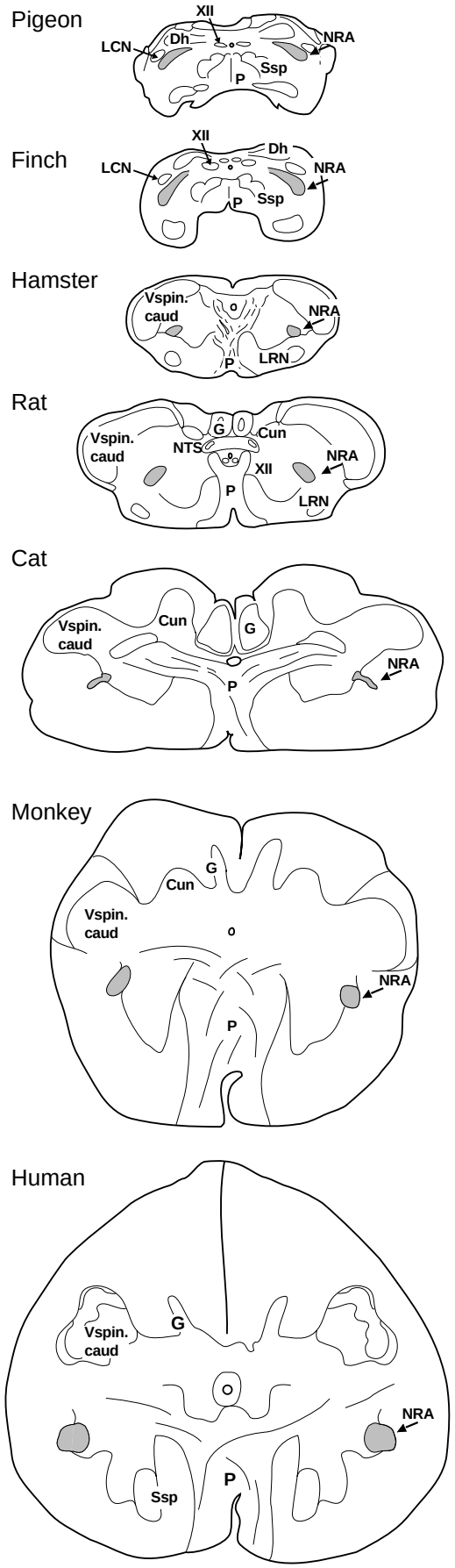
Behavior

Reproductive behavior is displayed by all species, but even between mammalian species there exist considerable variations in motor patterns. For example, female hamsters and rats only display the lordotic posture accompanied with hindlimb extension, and do not show treading with the hindlimbs which is so typical for the cat. Furthermore, receptive behavior in the hamster is very tonic and continues for several minutes after ending sensory stimulation. In rats, the lordotic posture is more "phasic" and lasts for only a few seconds.

Pathways

Differences in the organization of the CNS must underly these species differences in behavioral patterns. Indeed, subtle, but significant species differences appear to exist for the organization of the NRA-spinal, as well as for the spino-PAG pathways.

The NRA has been identified in humans (Olszewski and Baxter, 1954), monkey (Holstege, unpublished observations), cat (Merrill, 1970; Holstege, 1989), rat (Paxinos and Watson, 1986; Ellenberger and Feldman, 1990), hamster (Gerrits et al., in prep.), and bird (Wild, 1993; Fig. 6). In cat, monkey, and human, the NRA is relatively large and clearly protrudes into the white matter. In hamster and rat, the NRA is less pronounced, consisting of a rather diffuse group located among other neurons. Physiologically, cervical stimulation in female



rats has been shown to elicit activation of the psoas major and iliacus muscles (Martinez-Gomez et al., 1992), which are also involved in the lordosis posture in cats. Tracing studies in this laboratory indicate that the NRA-lumbosacral pathway exists in the hamster and rat (Kerstens et al., 1996; Gerrits et al., 1996). Moreover, in rat, cat, and hamster, these pathways seem to be organized differently, which is in line with the concept that differences in sexual behavior of these animals are caused by different NRA-lumbosacral motoneuronal projections.

Regarding the sensory pathways involved in reproductive behavior, lumbosacral-PAG projections have been demonstrated in monkey (Wiberg et al., 1987; Yeziersky, 1988; Zhang et al., 1990), cat (Wiberg and Blomqvist, 1984a; Yeziersky, 1988; VanderHorst et al., in press; Chapter 1; see Fig. 7), rat (Yeziersky, 1988), hamster (Gerrits et al., in prep.). In the monkey and to a lesser extent in the cat (see chapter 1), this pathway terminates in two distinct regions within the PAG, i.e. in the lateral part of the lateral PAG and adjacent tegmentum, and in the medial part of the lateral PAG. In contrast, in the rat and hamster the projections involve the entire lateral PAG and show no subdivisions.

Plasticity

In the rat and hamster, with estrous cycles of a few days, synaptic plasticity has been demonstrated in the ventromedial nucleus, arcuate nucleus, and the preoptic area which occurred in parallel with the time interval of the estrous cycle (Meisel and Luttrell, 1990; Frankfurt et al., 1990; Frankfurt and McEwen, 1991; Olmos et al., 1989; Langub et al., 1994). In the cat, with estrous intervals of 2-3 weeks up to a few months, plastic changes might be more prominent and might play a more important role than in animals with very short cycles. The estrogen induced sprouting in the NRA-lumbosacral pathway (chapter 5) supports this hypothesis. Seasonal breeders display their mating behavior only once a year, depending on the amount of light, the temperature, the availability of food and water, and social cues such as primer pheromones and the absence of stress (for review see Bronson and Heideman, 1994). These external cues influence neuronal cell groups in the hypothalamus,

Figure 6 Schamatic drawings of transverse sections through the lower brainstem, showing the NRA in the pigeon and the finch (Wild, 1993), hamster (Gerrits, VanderHorst and Holstege, in prep.), rat (Holstege, Kerstens, Moes, and VanderHorst, in press), cat (VanderHorst and Holstege, 1995), monkey (Holstege, unpublished), and human (Olszewski and Baxter, 1954).
Dh=dorsal horn; XII=Nu. hypoglossus; P=pyramidal tract; CL=lateral cervical nucleus; Ssp=supra-spinal nucleus; LRN=lateral reticular nucleus; NTS=nucleus of the solitary tract; G=gracile bucleus; Cun=cuneate nucleus; Vspin. caud=caudal spinal trigeminal complex

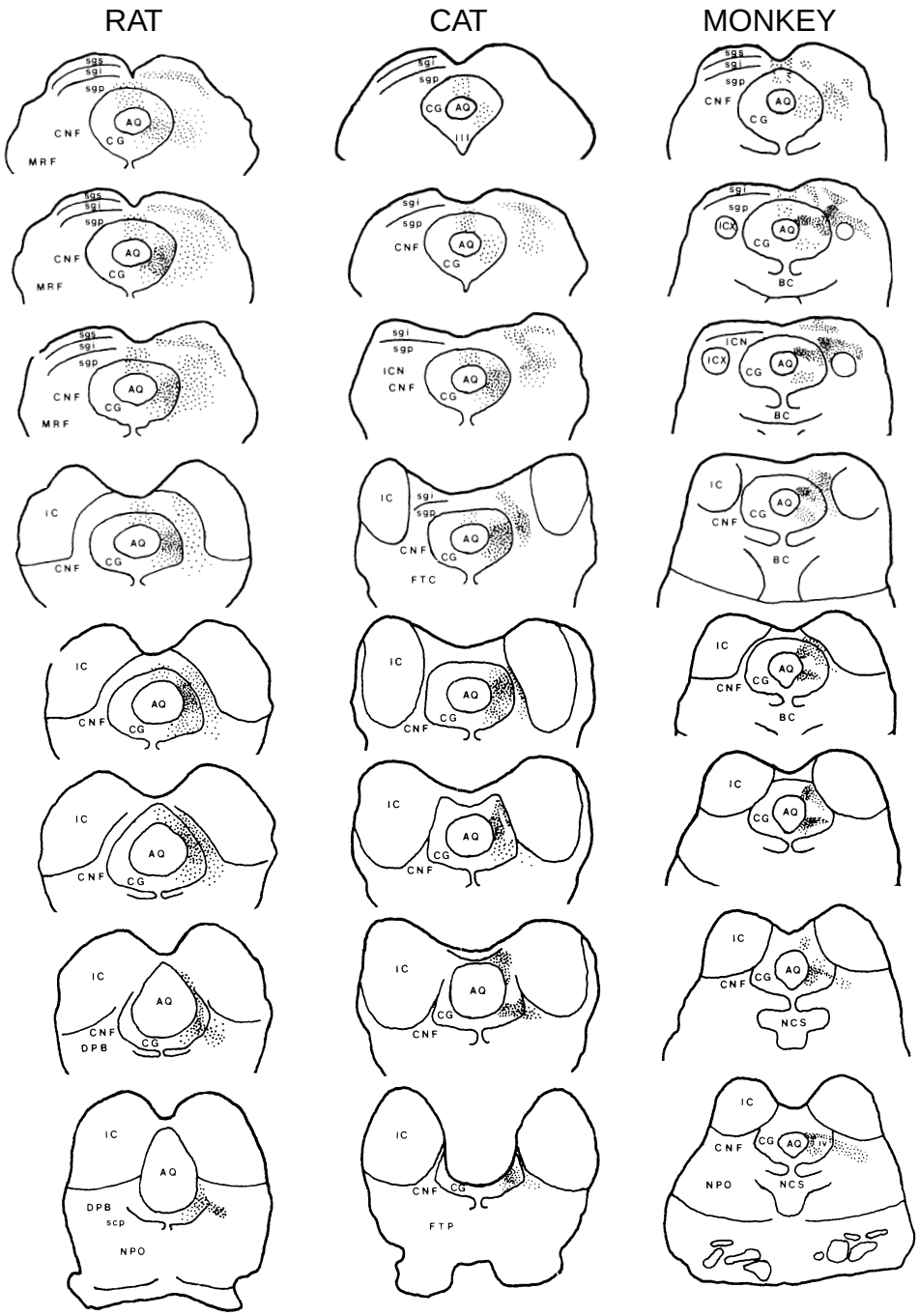


Figure 7 Termination pattern of lumbosacral-PAG projecting neurons in the rat, cat, and monkey (from Yezierski, 1988). Note that the projection is most diffuse in the rat, and most specific in the monkey.

which in turn change the release of LHRH from the pituitary, and thus the production of gonadal hormones (for review see Turek and Van Cauter, 1994). In these animals, plasticity as described for the NRA-spinal pathway in the cat might play a major role in preparing the CNS for reproduction.

Human reproductive behavior

The question remains how reproductive behavior is organized in humans. In all likelihood, the human species, like all other species, is equipped with a basic neural circuitry for sexual behavior. For example, vaginal stimulation in women, similar to the rat, has been shown to elevate the pain threshold (Whipple and Komisaruk, 1985). The major difference between humans and other animal species does not concern the basic cicuitry, but the facilitating and inhibitory effects of structures in the forebrain and cortex. Humans, with their large forebrains, are more capable than other mammals to control sexual behavior, as well as other emotional behaviors, such as anger.

Moreover, in contrast to other species, women are also

sexually active when the ovaries do not release estrogen, although gonadal hormones are well known for their effect on mood and performance (see Schreiner-Engel et al., 1981; Logue and Moos, 1988). Most other mammals only display this potentially risky mating behavior when conception is most probable. Humans are able to generate a safe environment, and apparently do not need this mechanism anymore.

The NRA as multifunctional interneuronal cell group for the emotional motor system

Multifunctional role of the NRA

NRA neurons have been shown to project directly to a distinct set of brainstem, and spinal cord motor nuclei (for review see VanderHorst and Holstege, 1996; see also Table 2 of chapter 3). The NRA is known to play a role in expiration (Merrill, 1970;1974), vomiting (Miller et al., 1987; 1995), defecation (Fukuda and Fukai, 1986; 1988), and vocalization (Holstege, 1989; Zhang et al., 1992; 1995). The anatomical results of this thesis strongly suggest that the NRA is also involved in reproductive behavior. In this context, the NRA appears to form a relay between the PAG and specific sets of somatic motoneurons involved in emotional behavior in general. It is very well possible that the NRA serves even more behaviors, such as the fetus expulsion reflex during parturition. In periparturient rats, electrical stimulation of the pelvic nerve has been shown to reflexively activate abdominal wall muscles (Cueva-Rolon et al., 1995), which reflex might be conveyed via the sacral-PAG-NRA pathway. The extensor responses of newborn rats during micturition are very much alike the lordotic posture (Beach, 1966; Williams, 1987; Williams and Lorang, 1987), and might be mediated by the same pathways.

The question is how the NRA is organized functionally or anatomically, in order to control these functions. Physiologically, it has been demonstrated that the NRA is capable to control completely different motor activities. For example, different populations of NRA neurons projecting to Onuf's nucleus are active during the retching and expulsion phase of vomiting (Miller et al., 1995).

Anatomically, a somatotopical, rostrocaudal organization for NRA-spinal neurons has not been revealed. In contrast, it has been shown that some NRA neurons project to both the C4-C6 and the L1-L3 segments (Portillo et al., 1994). These findings implicate that the NRA neurons are functionally organized, with for example the group of NRA-lordosis neurons located caudal to the group of NRA-vocalization neurons.

A similar organization might not only exist for the NRA-motoneuronal projections, but also for the PAG-NRA projections. This idea is supported by the findings that discrete tracer injections in the caudal NRA result in a distinct group of retrogradely labeled cells in the medial part of the lateral PAG (see introduction; VanderHorst and Holstege, 1996), whereas more rostral injections resulted in labeled neurons in the lateral PAG as well as in the adjacent tegmentum (Holstege, 1989). Other support comes from physiological studies in which vocalization and lordosis have been elicited by stimulation of the lateral and medial part of the lateral PAG, respectively (Sakuma and Pfaff, 1979a). These results indicate that indeed different groups of PAG-NRA neurons might be involved in the PAG-NRA-vocalization pathway and the PAG-NRA-lordosis pathway. Some overlap, however, cannot be excluded.

The NRA as relay for the emotional motor system

In general, spinal motoneurons receive their main input from interneurons in the spinal intermediate zone. Usually these projections are called propriospinal pathways. Supraspinal structures as well as afferent input from the periphery make use of these interneurons to influence the motoneurons. The propriospinal projections take part in the basic motor system in the concept of Holstege (Holstege, 1991, 1994; see introduction).

Specific, direct projections to distinct motoneuronal cell groups, bypassing the spinal interneurons, are rare. In the cat examples are Ia afferents from muscle spindles, and a very few rubrospinal fibers to C8-T1 digit motoneurons (Holstege, 1987; McCurdy et al., 1987).

NRA neurons also project directly to motoneurons, because they themselves are the premotor interneurons, similar to the ones in the spinal intermediate zone. Premotor interneurons of somatic (hindlimb) motoneurons usually receive peripheral Ia afferents or input from cortico- and rubrospinal tracts, but this is not the case for the premotor interneurons in the NRA. A probable explanation is that the NRA interneurons have a special function, i.e. they activate a set of motoneuronal cell groups in the lower brainstem and spinal cord, which activation has to be totally independent of voluntary input (from cortico- and rubrospinal pathways) or spinal reflexes. In this context, the NRA receives afferents from other sources such as the PAG (Holstege, 1989; Davis and Zhang, 1991; VanderHorst and Holstege, 1996), respiratory related neuronal cell groups in the pons and medulla, such as the NTS for vagal afferent input, Kölliker Fuse and lateral parabrachial nuclei (Bianchi and Barillot, 1975; Feldman, 1986 for review; Smith et al., 1989; Gerrits and Holstege, in press), and from two cell groups in the ventral medullary medial tegmental field (Gerrits and Holstege, in press).